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SOIL SCIENCE

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A METHOD FOR MEASURING CARBON DIOXIDE EVOLUTION FROM SOIL[1]

HARRY HUMFELD[2]

Bureau of Chemistry and Soils, U. S. Department of Agriculture

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In connection with studies on the microbiological activities in soils of different reaction as affected by the addition of green manures, it was deemed desirable to obtain some information on the rate of carbon dioxide evolution. A search of the literature for a suitable method for the determination of carbon dioxide from the soil revealed that, in general, the methods used may be divided into a number of distinct groups:

1. The soil sample is taken in a way intended to prevent the diffusion of air or to reduce it to a minimum. The sample is brought to the laboratory and analyzed for carbon dioxide content. The work of Leather (1) is an example of this type.

2. The soil air in a measured quantity is obtained from the soil in situ by means of a tube thrust or placed into the soil and the air obtained is analyzed in the laboratory. Russell and Appleyard's (6) and Potter and Snyder's (5) experiments illustrate this group of methods.

3. Soil samples are taken, brought into the laboratory, and carbon dioxide evolution is measured by placing the soil in a flask and passing carbon dioxide-free air over it. This method, used by Waksman and Starkey (7) and by many others, is essentially a measure of the carbon dioxide producing power of the soil under certain given conditions.

4. Marsh (4) modifies the preceding method in such a way as to pass air through the soil as well as over it.

5. Lundegardh (2) enclosed a sample of soil in a flask and measured the carbon dioxide accumulation in the flask air after 24 hours.

6. Lundegardh (3) later says that, methods which determine the carbon dioxide as it escapes from the soil by means of diffusion are in general, to be preferred to methods where an air stream passing through the soil is used. He therefore evolved an apparatus which consists of a "respiration bell" that is placed over an area of soil to collect the carbon dioxide as it escapes. At the end of 20 minutes a sample of the air under the bell is taken and analyzed. This, no doubt, gives results which approach natural conditions more closely than any of the other methods mentioned.

According to Lundegardh the rate of diffusion under these conditions is proportionate to time. Assuming that this is true, in soils of low organic activity, it very likely would not necessarily be true if the carbon dioxide evolution were considerably higher.

In our studies on decomposition of green manures under greenhouse conditions, preliminary tests showed that the amount of carbon dioxide evolution was 10, or more, times as great as that from untreated soil. Since it was be-

[1] Paper 332 of the outside publication series, Bureau of Chemistry and Soils.

[2] Assistant bacteriologist, office of soil microbiology.

lieved that such a rapid accumulation of carbon dioxide under the bell would tend to decrease the rate of diffusion, it seemed preferable to develop a method that would remove the carbon dioxide as fast as it diffused from the soil.

THE APPARATUS

The apparatus as finally developed consisted, therefore, of three essential parts—a collecting unit, an absorbing unit, and a source of suction.

The collecting unit

A rectangular galvanized iron box, 8 inches long, 3 inches wide, and 3 inches high, was used as a modified form of Lundegard's "respiration bell," small enough for use on the greenhouse plots. At each end of the box equidistant from the sides, a tube ¼ inch inside diameter, was inserted. One tube served for inlet, the other for outlet. The open side of the box was pressed down into the soil about one inch.

The absorption unit

The absorption unit consisted of a train of four bottles preceded by a flow-meter, which, in turn, consisted of a piece of capillary tubing 8 inches long and ¾ to 1 mm. in inside diameter, fastened in a U-shaped piece of glass tubing, 5 mm. in diameter, as shown in plate 1, figure 1. The flow-meter was fastened to a piece of wall board 12 inches square, and the U-shaped glass tubing was half filled with a colored mineral oil. The flow-meter was then calibrated in liters per hour against a West test meter. The flow-meter was connected with a piece of rubber tubing to the first bottle.

Wide-mouthed, approximately 1-liter bottles were used. The mouths were closed with two-hole rubber stoppers, each hole being fitted with a piece of glass tubing bent at right angles. The first bottle was empty and was used as a safeguard against dilution of the absorbent solution with moisture condensing from the air after passage over the soil. The second and third bottles each contained 800 cc. of 0.08 to 0.16 *N* KOH. Each of the absorption bottles was connected with a patented gas distributor consisting of a glass tube in which a disc made of ground sintered glass is fitted in such a way that the suction breaks up the air passing through the solutions into a great number of small bubbles. This device was found to be very efficient in promoting complete absorption. The fourth bottle was empty and served as a safeguard. It seemed also to facilitate adjustment of the rate of flow, since the air in this bottle acted as a cushion against rapid changes. The flow of air was regulated by means of a glass stopcock fastened in the line after the fourth bottle. A very delicate adjustment, however, was necessary.

The source of suction

A vacuum pump operated by an electric motor was used. A water aspirator was also found to give satisfactory results. Any number of units up to the

capacity of the pump could be connected together so that carbon dioxide evolution could be studied on a number of plots simultaneously.

As stated, the "respiration box" is placed in a suitable position on the soil. In order to obtain air of uniform carbon dioxide content, a rubber tube, 3 to 4 feet long, was fastened to the inlet of the box and tied up, with the opening about 3 feet above the surface of the soil. The outlet tube was connected by means of rubber pressure tubing to the calibrated flow-meter, which in turn was fastened by the same means to the glass tube in the first bottle. The bottles were connected together, the stoppers of the two bottles containing the KOH solution being provided with a glass trap. A form of trap used in the distillation apparatus for Kjeldahl nitrogen analyses was found satisfactory to prevent any mechanical carrying over of solution into the next bottle. The glass tube from the last bottle was then connected to the glass stopper in the suction line.

THE USE OF THE APPARATUS

It was found that 10 liters of air an hour was sufficient to carry off the carbon dioxide from the soil, even at the highest rate of evolution encountered. Lower rates could be used with smaller amounts of carbon dioxide, but it was found that with smaller rates of flow the adjustment to keep the flow steady was more difficult.

As the apparatus was used, the KOH solution in the first bottle absorbed all of the carbon dioxide, the second bottle acting as a check on the completeness of absorption. When it was found that the carbon dioxide had neutralized the KOH solution to such an extent that the next absorption period would reduce the strength to less than one-half the original strength, a bottle containing new KOH solution was inserted in its place. As a rule, samples were taken and titrated every 24 hours.

After a number of variations, the titration was finally standardized so that now it is as follows: hydrochloric acid was made up of such a normality (0.08336 *N*) that 1 cc. was equivalent to 0.5 mgm. carbon as carbon dioxide. The KOH solution was made up to approximately the same strength or any strength up to twice this strength, depending upon the rapidity of carbon dioxide evolution expected. Each 24 hours a 25-cc. aliquot was taken from each bottle in each unit.

These aliquots were brought to boiling and titrated first to the endpoint of phenolphthalein and then to the endpoint of methyl orange. Aliquots of the original KOH solution were titrated in a similar manner and the amount of the titration from the endpoint of phenolphthalein to the endpoint of methyl orange was subtracted from the corresponding titration of the aliquots taken after absorption. The number of cubic centimeters of hydrochloric acid required, divided by 2, gave the milligrams of carbon as carbon dioxide in the aliquot. From this, the carbon dioxide evolution for the area of the box or for any unit area could be calculated. It was found that dilution of the ali-

quots with 100 cc. distilled water before heating gave better endpoints for phenolphthalein.

In order to eliminate any possibility of suction of air from the soil, instead of freeing the air from carbon dioxide before passing it over the soil, a blank determination was run. The net amount of carbon dioxide obtained from the soil was then determined by deducting the amount of this blank determination. The calculation then was as follows:

The number of cubic centimeters of hydrochloric acid for the phenolphthalein titration is deducted from the number of cubic centimeters required for the

TABLE 1

Typical titrations for a 24-hour period on plots in the greenhouse under different treatments

(C as CO_2 = mgm. in 24 hours)

PLOT NUMBER	GREEN MANURE USED	HCl REQUIRED†		DIFFERENCE MINUS BLANK TITRATION‡ C AS CO_2	C AS CO_2 PER BOX; IN 24 HOURS
		Phenolphthalein	Methyl orange		
		cc.	*cc.*	*mgm.*	*mgm.*
L 1	None	40.0	44.7	1.3	17.2
L 2*	None	39.6	44.7	1.5	24.0
L 3	Rye	31.3	44.7	5.7	106.8
L 4*	Rye	36.9	44.7	2.9	67.2
L 5	None	40.2	44.7	1.2	14.4
L 6*	None	37.6	44.6	2.4	51.2
L 7	Vetch	37.5	44.7	2.6	57.6
L 8*	Vetch	35.4	44.7	3.6	91.2
L 9	Rye	36.5	44.7	3.1	73.6
L 10*	Rye	34.6	44.6	4.0	102.4
L 11	Vetch	38.0	44.7	2.3	49.6
L 12*	Vetch	35.4	44.7	3.6	91.2
Air	Control	44.1	44.7	0.8	25.6§

* All even-numbered plots had been limed and were neutral in reaction, whereas the odd-numbered plots were acid.

† 25-cc. aliquots titrated.

‡ Blank titration equals 2 cc.

§ This figure has been deducted from each preceding figure in this column.

methyl orange titration. This gives the number of cubic centimeters actually used by the carbon dioxide absorbed.

The original KOH solution, however, required a certain amount of hydrochloric acid to change the reaction from the endpoint of phenolphthalein to the endpoint of methyl orange. This amount, which was 2 cc. in the KOH solution used, is deducted from the apparent difference. The figures are given in the column headed "Difference minus blank titration," table 1. This figure, multiplied by the number of cubic centimeters of KOH solution in the absorption bottle and divided by half the number of cubic centimeters in the aliquot taken for titration, gives the milligrams of carbon dioxide absorbed from the area of soil covered by the respiration box.

For plot L4, in table 1, the number of milligrams of carbon dioxide would be

$$\frac{44.7 - 40.0 - 2.0}{2} \times \frac{800}{25} = 43.2$$

If it is desired, the milligrams of carbon dioxide absorbed from the carbon dioxide in the air, given in the last line of table 1, can be deducted. The net results in this case would be

$$43.2 - 26.0 = 17.2 \text{ mgm. carbon dioxide.}$$

The method described was used in the determination of carbon dioxide evolution under greenhouse conditions.

FIELD EXPERIMENTS

Subsequently it was decided that trials with the same apparatus under field conditions would be advisable. Accordingly the apparatus was set up in the field on some experimental plots on Keysport clay loam. Two units were placed on each of three plots. One plot was kept fallow, another plot had a heavy growth of soybeans, and the third plot was growing a crop of corn broadcast for green manure. At the start of the carbon dioxide determination the crops were fully grown and ready to be turned under. The soil was exceedingly dry, however, and it was considered inadvisable to turn the corn and soybeans under before a rainfall had occurred. The results obtained for a number of days therefore indicate the amount of carbon dioxide given off by the soil under very low moisture content.

A rain occurred on September 5 and 6 and the crops were plowed under on September 7. However, the moisture in the soil was still far from optimum, therefore, the table given also shows the time and amount of rainfall during the experiment.

The first data obtained showed that only small amounts of carbon dioxide were being given off. It was essential, therefore, that chemical determination be as accurate as possible. The concentration of KOH was therefore reduced so that it was only about 0.04 to 0.05 normal.

When the titrations were made it was noticed that the endpoint of phenolphthalein was too indefinite, because of the loss of carbonate from the solution as carbon dioxide; this caused the reappearance of the red color of phenolphthalein. In order to overcome this difficulty the carbonate in the solutions was precipitated as barium carbonate by adding an excess of barium chloride to the samples taken for titration.

The procedure was standardized as follows:

Two hundred cubic centimeter of each sample were placed in tall glass cylinders, an excess of approximately normal barium chloride (5 to 20 cc. was usually required) was added, and the volume made up to 250 cc. with distilled water. The cylinders were then stoppered and the solutions were shaken. Time was allowed for the precipitate to settle and when the supernatant solution was clear two 25- or 50-cc. aliquots were pipetted off and titrated.

The original KOH solution was treated in a similar manner and the number of cubic centimeters of HCl required for the original KOH solution minus the number of cubic centimeters required for the solutions after CO_2 was passed through them, gives the number of cubic

TABLE 2

Amount of carbon as carbon dioxide given off in 24 hours from soil under different treatments—September 24, 1929

PLOT NUMBER	TREATMENT	BOTTLE	ALIQUOT TAKEN FOR TITRATION	HCl REQUIRED		DIFFERENCE USED BY CO_2	C AS CO_2 FOR AREA OF BOX	MINUS AMOUNT IN AIR	C AS CO_2 PER SQ.M.	RATE OF AIR HOURLY
					Average					
			cc.	*cc.*	*cc.*	*cc.*	*mgm.*	*mgm.*	*gm.*	*liters*
K6W	Soybeans	1	25	33.25	33.22					
			25	33.20		5.48	109.6	54.4	3.52	13
		2	25	38.72	38.70	0.00	0.0			
			25	38.68						
K6W	Soybeans	1	25	33.20	33.15	5.55	111.0	57.0	3.68	13
			25	33.10						
		2	25	38.60	38.64	0.06	1.2			
			25	38.68						
K4E	Fallow	1	50	15.99	15.05	6.15	61.5	31.8	2.05	7
			50	16.11						
		2	50	21.10	21.20	0.00	0.0			
			50	21.29						
K4E	Fallow	1	50	14.60	14.66	6.54	65.4	27.2	1.76	9
			50	14.72						
		2	50	15.65	15.74	0.00	0.0			
			50	15.82						
K7E	Corn	1	25	33.29	33.14	5.56	111.2	59.8	3.87	13
			25	32.99						
		2	25	38.55	38.55	0.15	3.0			
			25	38.54						
K7E	Corn	1	25	32.72	32.74	5.96	119.2	67.8	4.38	13
			25	32.77						
		2	25	38.53	38.56	0.14	2.8			
			25	38.60						

centimeters of HCl equivalent to the CO_2 absorbed. These figures, multiplied by the proper factor to obtain the amount in the total amount of solution and divided by 2, since each cubic centimeter of HCl is equivalent to 0.5 mgm. of C as CO_2, give the mgm. of C as CO_2 obtained from the area of soil under the respiration box.

TABLE 3

Carbon as carbon dioxide evolved under field conditions

(Carbon as CO_2 in gm. per sq. m.)

	AUG. 30	AUG. 31	SEPT. 1	SEPT. 2	SEPT. 3	SEPT. 4	SEPT. 5	SEPT. 6	SEPT. 7	SEPT. 8	SEPT. 9	SEPT. 10	SEPT. 11	SEPT. 12
Soybeans	2.32	1.97	2.04	1.87	1.78	1.81	1.03	6.28	....	13.26	14.25	15.86	....	8.54
	2.39	2.11	1.71	1.94	1.35	1.71	1.07	4.92	....	9.38	7.72	11.35	....	21.58
Average.....	2.36	2.04	1.86	1.90	1.56	1.76	1.05	5.60	....	11.32	10.98	13.60	....	15.06
Fallow	3.32	3.04	2.07	....	2.71	1.71	2.05	4.43	....	2.10	3.05	3.05	....	2.32
	3.39	3.17	2.04	....	2.63	1.87	1.42	9.34	....	4.55	3.79	3.32	8.88	7.61
Average.....	3.36	3.10	2.05	....	2.67	1.79	1.73	6.88	....	3.37	3.42	3.18	8.88	4.96
Corn	.....	1.42	1.19	0.60	....	....	4.56	2.00	....	7.13	7.63	10.55	4.23	7.16
	.....	1.23	.....	0.56	0.42	0.52	1.86	6.78	....	9.28	11.40	16.15	9.50	11.60
Average.....	.....	1.33	1.19	0.58	0.42	0.52	3.21	4.39	....	8.20	9.02	13.35	6.86	9.38
Rainfall, inches.....	.....	.....	.....	....	....	.....	0.07	0.48	....	.....	0.04	0.80	....	....

	SEPT. 13	SEPT. 14	SEPT. 15	SEPT. 16	SEPT. 17	SEPT. 18	SEPT. 19	SEPT. 20	SEPT. 21	SEPT. 22	SEPT. 23	SEPT. 24	SEPT. 26	SEPT. 28
Soybeans	10.17	13.15	12.32	6.14	5.72	17.45	8.04	4.16	3.63	5.86	2.09	3.52	3.12	4.52
	20.30	21.00	13.60	6.03	7.25	17.05	12.78	5.53	4.44	5.62	2.99	3.68	3.88	4.61
Average.....	15.24	17.07	12.96	6.08	6.48	17.25	10.41	4.84	4.03	5.74	2.54	3.60	3.50	4.56
Fallow	3.70	3.99	3.23	0.94	2.67	4.47	1.51	1.66	2.24	2.64	1.59	2.05	1.34	2.86
	5.20	6.47	5.49	1.95	1.88	8.06	9.63	3.15	2.84	2.48	2.50	1.76	2.42	2.10
Average.....	4.45	5.23	4.36	1.45	2.27	6.26	5.56	2.40	2.54	2.56	2.04	1.90	1.88	2.48
Corn	3.04	10.60	9.58	3.53	6.60	12.56	12.07	5.25	4.92	....	....	3.86	3.19	4.23
	4.93	11.48	18.90	10.03	14.62	25.60	24.70	11.07	5.78	4.25	4.02	4.31	5.64	5.78
Average.....	3.98	11.04	14.24	6.76	10.61	19.08	18.38	8.16	5.35	4.25	4.02	4.08	4.41	5.00
Rainfall, inches.....	.....	0.44	.....	....	0.29	0.28	.....	....	....	....	....	....	.....	.....

The number of mgm. for the area under the box multiplied by 64.6 and divided by 1,000 gives the number of grams of carbon as carbon dioxide given off by each square meter of soil area.

Table 2 gives an example of a typical series of titrations, and shows the accuracy of the method. Results show that the greatest variations obtained on duplicate boxes on the same plots are due to differences caused by activities in the soil and that as far as the analytical operations are concerned, very good results can be obtained.

Table 3 shows what results may be obtained under field conditions, as described, when green manure is turned under. This table shows the amount of carbon as carbon dioxide given off, both before and after the green manures were turned under, and the effect of the decomposition of soybeans and of corn on the carbon dioxide evolution.

The crop was rolled down flat and plowed under as would be done under actual field operations. The respiration boxes were then placed on the surface of the soil at random so that any differences in determinations would be due to variations in the amount and nature of the organic matter below them.

The results show that although in a general way the fallow plot gives fairly good agreements between duplicates, the results obtained from duplicates on the soybean and the corn plots show considerable differences after the crops were turned under.

As moisture in this case was no doubt a limiting factor, an increase was shown in carbon dioxide evolution wherever a rainfall occurred in sufficient amount to cause a definite increase in the soil moisture. This is true of the fallow plots as well as of the soybeans and the corn plots.

In adapting the method to field conditions, it was found that it was impossible to keep the flow of air in all the units at 10 liters an hour, without variation. This difficulty was partly overcome by using a safety valve, consisting of a large glass test tube partly filled with mercury and fitted with a 2-hole rubber stopper provided with two bent glass tubes, one of which reached down into the mercury. This safety valve was connected with a bent glass tube fitted into the stopper of the fourth bottle in the absorption unit, that is, the bottle next to the source of suction.

The rate was then more satisfactorily regulated by opening the stopcock in the suction sufficiently to allow a somewhat greater amount of air to pass than the required 10 liters an hour. Then by moving the long glass tube in the safety valve up or down the rate could be cut down to the required amount. Some variation still took place during the 24 hours between samplings, but adjustments could be made much more readily.

It had been observed, also, that as the air passed from the respiration box, through the flow-meter and into the absorbing bottles, a certain amount of moisture condensed, especially in the flow-meters. This diluted the oil in the flow-meters and necessitated renewal of the oil from time to time.

In order to overcome this an empty bottle was inserted between the box and the flow-meter. Indications are that this modification eliminates the moisture accumulation or at least reduces it to a minimum.

Plate 1, figure 3 shows the absorption unit as it is now used in the field.

The method as now employed seems to be adapted to the measurement of carbon dioxide evolution from the soil or from material incorporated with the soil, assuming that diffusion from the soil is a direct result of activities resulting in the liberation of carbon dioxide.

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PLATE 1

The Carbon Dioxide Apparatus

Fig. 1. A unit of the carbon dioxide apparatus.
Fig. 2. General view of greenhouse plots and units of carbon dioxide apparatus.
Fig. 3. A Unit of the Carbon Dioxide Apparatus Set up in the Field.

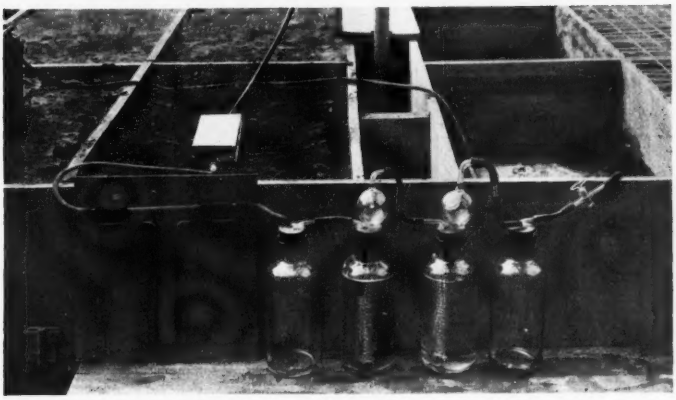

FIG. 1

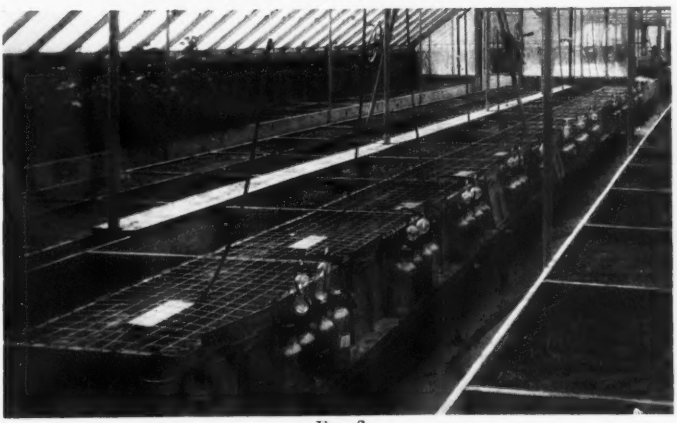

FIG. 2

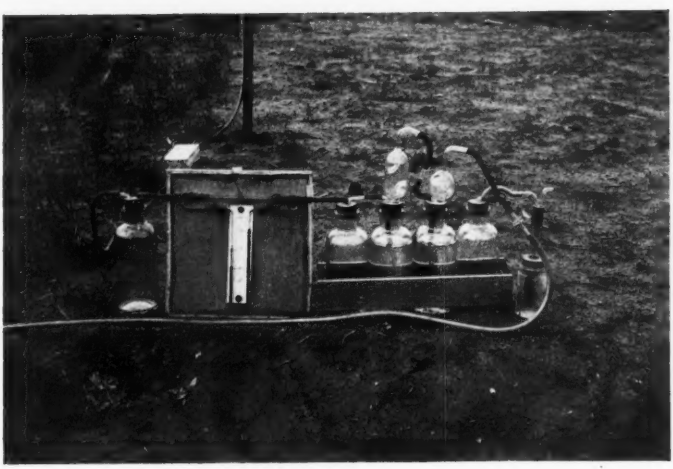

FIG. 3

PHOSPHATE STUDIES IN SOLUTION CULTURES[1]

J. W. TIDMORE[2]

Alabama Agricultural Experiment Station

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The question of minimum phosphate concentration for maximum plant growth should be of particular interest since there are many soils reported which have extremely low phosphate concentration in the soil solution as obtained by the method described by Burd and Martin (1). Parker and Tidmore (9) and Pierre and Parker (12) cited soil solutions containing as little as 0.02 p.p.m. inorganic PO_4, and some of these soils produced in the field as much as 45 bushels of corn an acre.

Considerable work has been done during the past few years concerning the minimum concentration of phosphate required for maximum growth of plants in solution cultures. Hoagland and Martin (4) obtained a satisfactory growth of barley in culture solutions containing 0.7 p.p.m. PO_4, and a maximum growth at 1.1 p.p.m. PO_4. Parker (8) obtained maximum growth of corn and soybeans in culture solutions having a phosphate concentration of 0.5 p.p.m. Subsequently, Parker and Pierre (11) obtained a maximum growth of corn at a phosphate concentration of 0.25 p.p.m. They expressed the opinion that a very good growth could be obtained at a phosphate concentration of 0.1 p.p.m., if that concentration could be actually maintained.

In the culture solution experiments mentioned in the foregoing, the phosphate concentrations were not adequately maintained. The quantity of the culture solution used for each culture was entirely too small for the number of plants grown in it. Hoagland and Martin (4) used 4-liter containers for one plant whereas Parker and Pierre (11) used 100-liter vessels for three plants. They state that with that volume the PO_4 concentration was not adequately maintained. It seemed desirable, therefore, to make a further study of plant growth in culture solutions of varying phosphate concentrations.

[1] A thesis submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in plant nutrition in the University of California. This investigation was suggested by Doctor F. W. Parker, division of agronomy and soils, Alabama Experiment Station, to whom grateful acknowledgment is made for advice and criticism throughout the investigation. The writer also wishes to express his appreciation for the helpful suggestions tendered by Prof. D. R. Hoagland, division of plant nutrition, University of California. Published with the permission of the director of the Alabama Agricultural Experiment Station.

[2] Associate professor of agronomy and soils, Alabama Agricultural Experiment Station.

In the following experiments a very large volume of solution with relatively few plants was used for each culture. The phosphate concentration was practically maintained. The following points were studied in the investigation:

1. The rate of phosphate absorption by plants grown in solutions of various phosphate concentrations.
2. The influence of low phosphate concentrations on the total plant growth and on the rate of growth.
3. The phosphate concentration and buffer capacity of the sap of plants grown in solutions of various phosphate concentrations.
4. The influence of the reaction of the culture solution on growth and on phosphate absorption.

EXPERIMENTAL PROCEDURE

Phosphate concentrations of 0.05, 0.1, 0.2, and 0.5 p.p.m. were maintained constant in the various cultures by using a large volume of solution for each plant and renewing the PO_4 content of the solution from one to three times a day. Nine plants were grown in a 1,000-liter culture vessel. To continually

TABLE 1

Composition of the culture solution

	K	Ca	Mg	NO_3	SO_4	B	NaCl	$MnSO_4$
Mgm. equivalent	0.76	1.90	0.81	2.61	0.81	....	....	.....
P.P.M.	29	38.17	9.86	162.1	39.0	0.25	0.60	0.375

renew the solution about the plant roots and to aerate the cultures, the solution was thoroughly stirred by bubbling a strong stream of air through it.

In order to renew the phosphate concentration of the culture solutions, the PO_4 was determined by using a small aliquot of each solution; then sufficient potassium acid phosphate was added to give the desired PO_4 concentration. During the growing period, the culture solutions with 0.05, 0.10, 0.20, and 0.50 p.p.m. PO_4 had an average minimum concentration of 0.048, 0.086, 0.147, and 0.440 p.p.m. PO_4, respectively, as determined by the colorimetric method described by Parker and Fudge (10).

The culture solution used in the experiments was one-fourth the concentration of Hoagland's (4, p. 372) with the addition of a small amount of boron. Table 1 gives the composition of the culture solution. Iron was supplied as ferric tartrate, as described by Parker (8). The plants were given iron about once each week, or whenever a slight chlorotic condition indicated a deficiency.

In experiment 1, the height of the plants, measured from the base of their stem to the tip of the longest leaf, was determined each week to indicate the rate of growth. The green weight of the plants was also obtained each week in experiment 2 after removing the plants from the culture solutions and allowing the root system to drain.

After the plants were harvested, they were weighed and a portion was placed in a cold storage room (20°F.) for freezing so that the plant sap could be obtained by means of a screw press. The concentration of PO_4 in the sap was determined and buffer curves were made. After the dry weight of the plants was determined, they were ground and the percentage PO_4 was determined by the use of Fiske and Subbarow's method as described by Parker and Fudge (10).

EXPERIMENT 1

Corn, sorghum, and tomato seeds were germinated in quartz sand and transferred, when 1 week old, to the culture solutions containing different phosphate concentrations. Three seedlings of each crop were grown in each culture solution for 46 days. Each treatment was in duplicate.

While the plants were being grown in the culture solutions, similar plants were grown in 4-gallon soil cultures of a sandy loam fertilized at the rate of

TABLE 2

PO_4 absorbed, during one week, by plants growing in culture solutions of the phosphate concentrations indicated

ABSORPTION PERIOD	PO_4 (P.P.M.) IN CULTURE SOLUTIONS			
	0.05	0.1	0.2	0.5
weeks	*mgm.*	*mgm.*	*mgm.*	*mgm.*
3	32	60	260	440
4	57	214	460	1,060
5	34	328	633	1,236
Total.....	123	602	1,352	2,736

1,000 pounds of superphosphate, 100 pounds of muriate of potash, and 500 pounds of sodium nitrate to the acre. There were duplicate cultures with two plants each.

Rate of PO_4 absorption

The amount of PO_4 absorbed from each culture solution was determined for three weeks, beginning when the plants were 3 weeks old. The results, given in table 2, represent the average of duplicate cultures, which agreed rather closely. It may be seen from table 2 that the phosphate absorbed is almost directly proportional, with few exceptions, to the concentration of PO_4 in the culture solution. The plants were larger and had a more extensive root system in the culture solutions at higher phosphate concentrations. This would account, in part, for an increased PO_4 absorption as the phosphate concentration of the culture solution increased.

Rate of growth and crop yields

The average height of these plants was determined each week during the growing period beginning when the plants were about 3 weeks old. The weekly increase in height is shown in table 3. The rate of growth, as measured by the height of the plants, grown in solutions having a phosphate concentration of 0.1, 0.2, and 0.5 p.p.m. PO_4 is about the same. Corn plants grown at 0.2 and 0.5 p.p.m. PO_4 increased in height each week an average of 35 cm., whereas those at 0.1 p.p.m. increased 28 cm. This difference in rate of growth is probably insignificant. This method of determining the rate of growth may be misleading because the diameter of the stems increased with the PO_4 concentration of the culture solution. It would seem that a determination of the weight of the plants at intervals would give a better indication of the rate of

TABLE 3

Average weekly increase in height of the plants grown in culture solutions at PO_4 concentrations indicated

AGE OF PLANTS	CORN				SORGHUM				TOMATO			
	PO_4 (p.p.m.) in culture solution				PO_4 (p.p.m.) in culture solution				PO_4 (p.p.m.) in culture solution			
	0.05	0.10	0.20	0.50	0.05	0.10	0.20	0.50	0.05	0.10	0.20	0.50
weeks	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*
2	30	36	41	57	14	16	18	24	8	11	11	13
3	11	24	35	37	8	23	24	27	5	7	12	10
4	11	28	31	32	9	19	27	22	6	11	11	10
5	21	28	39	34	11	23	30	27	8	12	11	14
6	19	34	34	41	15	41	50	59	...	....	....	....
Average...	15.5	28.5	34.7	36.0	10.7	26.5	32.7	33.7	6.3	10.0	11.3	11.3

growth. The corn plants in culture solutions containing 0.05 p.p.m. PO_4 increased in height each week only 15 cm. This shows that the corn plants at 0.05 p.p.m. PO_4 made a much slower growth than did those at higher concentrations. The rate of growth in each culture was practically constant, as determined by measuring the plants. The same general trend holds for sorghum.

The increase in height of tomato plants each week was 6, 10, 11, and 11 cm., grown in culture solutions containing 0.05, 0.1, 0.2, and 0.5 p.p.m. PO_4, respectively.

The most interesting result of this experiment is set forth in table 4, which gives the dry weights of the plants grown at various phosphate concentrations. To obtain these weights the plants were harvested and dried in an oven at 70°C. for two days. Three plants were weighed together, because the roots were entangled and separation was not feasible. Table 4 shows the average weight of three plants; the duplicates agreed as well as could be expected.

Contrary to the findings of Parker and Pierre (11), in this study the corn plants which were grown in culture solutions having a PO_4 concentration of 0.2 p.p.m. weighed about 65 per cent as much as those grown at 0.5 p.p.m. PO_4. Corn made very little growth in solution cultures with 0.05 p.p.m. PO_4. It will be noticed that the corn (roots and tops) grown at 0.05 p.p.m. weighed approximately one-third as much as those grown at 0.1 p.p.m. PO_4. The data indicate a decided increase in corn growth as the phosphate concentration of the culture solution increased. The top:root ratio increased with the phosphate concentration of the culture solution. This would indicate that the root growth was relatively larger than the top growth at low phosphate concentrations.

As shown in table 4, corn grown in the soil cultures, which were well fertilized, made very little more growth than those grown in the culture solutions at 0.1 p.p.m. PO_4. The root growth was smaller in the soil cultures than in the culture solutions at 0.1 p.p.m. PO_4. Of course, the weight of the root system

TABLE 4

Dry weight of 3 plants grown in culture solutions with PO_4 concentrations indicated

PO_4	CORN			SORGHUM			TOMATOES		
	Tops	Roots	Total	Tops	Roots	Total	Tops	Roots	Total
p.p.m.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0.05	10.9	7.1	18.0	7.0	4.1	11.1	10.6	4.3	14.9
0.10	38.7	19.2	57.9	26.0	9.9	35.9	31.8	7.7	39.5
0.20	84.2	24.6	108.8	42.1	11.5	53.6	40.5	9.8	50.3
0.50	136.9	30.4	167.3	48.0	12.9	60.9	43.2	7.8	51.0
Soil	55.2	13.4	68.6	32.9	5.6	38.5	49.8	9.1	58.9

may not necessarily be a measure of the absorbing surface. The table shows that sorghum and tomatoes made as good growth in culture solutions at 0.2 p.p.m. as at 0.5 p.p.m. PO_4. In most cases the differences are too small to be significant. It is claimed that these crops require a large amount of phosphate, yet they practically made a maximum growth at 0.2 p.p.m. PO_4. The growth of sorghum and tomatoes in soil cultures was about the same as in culture solutions at 0.2 p.p.m. PO_4.

PO_4 content of plant and plant sap

After the plants were dried and ground, the percentage PO_4 was determined by the Fiske and Subbarow method, as described by Parker and Fudge (10). The results, given in table 5, show that in general the PO_4 content of the plants increases with increasing concentrations of PO_4 in the culture solutions. The magnitude of the increase was greatest in sorghum and smallest in tomatoes. With all three crops the increase in PO_4 content was greater in the tops

than in the roots. In the case of corn and sorghum grown at concentrations of 0.05 and 0.1 p.p.m. PO_4, the PO_4 content of the roots was higher than that of the tops. The opposite relation existed when the PO_4 concentration of the culture solution was increased to 0.2 and 0.5 p.p.m. At all concentrations of PO_4 the roots of the tomato plant had a higher percentage of PO_4 than the tops.

It is of interest to note that the PO_4 content of the tomato plants was uniformly much higher than that of the corn and sorghum. This might be expected to indicate that the tomato would make poorer growth in solutions of low PO_4 content than would corn or sorghum. Such, however, is not the case, as is indicated by the yields recorded in table 4.

TABLE 5

PO_4 content of dry tissue of plants grown in culture solutions with PO_4 concentrations indicated

PO_4 IN CULTURE SOLUTION	CORN		SORGHUM		TOMATOES	
	Tops	Roots	Tops	Roots	Tops	Roots
p.p.m.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.05	0.33	0.46	0.32	0.46	0.52	0.80
0.10	0.69	0.68	0.61	0.59	0.56	0.93
0.20	0.89	0.75	0.97	0.85	0.72	1.34
0.50	1.11	0.89	1.24	1.04	1.22	1.65

TABLE 6

PO_4 content of sap from plants grown in culture solutions with phosphate concentrations indicated

PO_4 IN CULTURE SOLUTION	CORN		SORGHUM		TOMATOES	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
0.05	167	76	183	108	152	86
0.10	185	55	320	68	204	118
0.20	735	68	610	185	270	180
0.50	1,200	196	1,000	430	420	320

As previously indicated, the plant sap was obtained by means of a screw press after the plants had been frozen and permitted to thaw. The sap was then centrifuged until it was perfectly clear. The centrifuging required only about five minutes. The phosphate determinations were made in duplicate by the colorimetric method. Table 6 shows that the PO_4 content of the sap from the leaves and stems increased with increasing concentrations of PO_4 in the culture solutions. The increase was greatest in corn and smallest in tomatoes. The PO_4 concentration in sap from the leaves was higher than that from the stems in all cases. The sap of corn leaves at maximum growth had a much higher PO_4 content than that of sorghum or tomatoes, whereas that from the corn stems had a lower PO_4 content than that from sorghum or tomatoes.

EXPERIMENT 2

This experiment was planned to study in more detail points brought out in experiment 1. Six corn, three sorghum, and three tomato plants were grown for 76 days in each 1,000-liter culture vessel. The culture solutions used had the same composition as those used in experiment 1. Studies were made of PO_4 absorption, rate of growth, total yield of dry matter, and PO_4 content of plant and plant sap. In addition, a study was made of the influence of PO_4 content of the sap on its buffer capacity.

Corn and sorghum were grown in soil cultures at the same time as the plants in the solution cultures. A quantity of soil was obtained from plot 2 of the Cullars' Rotation; this soil is very low in phosphate. The soil was placed in 4-gallon earthenware pots. All of the pots were fertilized with muriate of

TABLE 7

*Amount of phosphate absorbed, in weekly periods, from culture solutions of the PO_4 content indicated**

ABSORPTION PERIOD	PO_4 (P.P.M.) IN CULTURE SOLUTIONS			
	0.05	0.10	0.20	0.50
	mgm.	*mgm.*	*mgm.*	*mgm.*
First week	82	100	165	410
Second week	16	42	80	190
Third week	38	28	38	60
Fourth week	27	42	84	370
Fifth week	22	66	252	860
Sixth week	29	118	504	1,445
Seventh week	8	232	812	2,110
Eighth week	0	326	704	2,350
Ninth week	0	205	725	2,150

* The plants were 7 days old when the absorption study was started.

potash (100 pounds an acre) and sodium nitrate (500 pounds an acre). Half of these soil cultures were fertilized with superphosphate (1,000 pounds an acre) and are designated as high phosphate cultures. The other half of the soil cultures received no phosphatic fertilizer and are designated as low phosphate cultures. These soil cultures were included to compare the growth obtained in soil cultures containing high and low phosphate with the growth in culture solutions.

Rate of PO_4 absorption

The influence of PO_4 concentration of the culture solution on the rate of absorption of PO_4 by plants was studied by determining the amount absorbed each day for nine weeks (table 7).

The amount of PO_4 absorbed during the first week was higher than that

absorbed during the second and third weeks, probably because of the change in the root system of the corn and sorghum plants. The plants were 2 weeks old after they had been in the culture solutions one week. At this time there is a gradual disappearance of the temporary root system and a beginning of the permanent root system.

The PO_4 absorbed from the culture solutions increased with the phosphate concentration of the solutions, but the rate was not directly proportional to the concentration. In most cases the PO_4 absorbed was more than doubled as the PO_4 concentration of the culture solution increased. The plants, however, were larger and had a better developed root system in the culture solutions with high PO_4 concentrations than did those at low PO_4 concentrations. This would afford more absorbing surfaces. The plants grown in the culture solutions at 0.05 p.p.m. PO_4 absorbed very little PO_4 during the seventh week

TABLE 8

The average weekly increase in height of plants grown in culture solutions at PO_4 concentrations indicated

GROWTH PERIOD	CORN				SORGHUM			
	PO_4 (p.p.m.) in culture solutions				PO_4 (p.p.m.) in culture solutions			
	0.05	0.10	0.20	0.50	0.05	0.10	0.20	0.50
weeks	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*
2	7.5	7.5	8.1	7.6	8.8	7.7	10.0	10.0
3	19.5	22.3	23.9	24.4	3.2	5.3	5.0	5.0
4	7.0	10.0	14.0	25.0	1.0	2.0	3.0	8.0
5	0.0	13.0	30.0	41.0	0.0	6.0	15.0	18.0
6	1.0	19.0	27.0	36.0	0.0	11.0	20.0	25.0
7	0.0	21.0	52.0	45.0	0.0	12.0	23.0	41.0
8	0.0	20.0	24.0	38.0	0.0	21.0	31.0	35.0
Average....	4.6	17.6	28.5	34.9	0.7	9.5	16.1	22

and none thereafter, because they were practically dead. It is shown that the phosphate concentration of the culture solution has a decided influence on the rate at which PO_4 is absorbed by plants.

Rate of growth and yields

The average height of the plants grown in culture solutions with the various PO_4 concentrations was determined each week, beginning after the plants had been in the culture solutions two weeks. The results for corn and sorghum are presented in table 8. When the corn plants had been in the culture solution two weeks they were fairly uniform in height (table 8). During the third week the difference in rate of growth was small. After this period the rate of growth increased with the increased PO_4 concentration of the nutrient solution. The corn grown at 0.05 p.p.m. PO_4 made no growth after the fourth

week. The rate of growth of the corn plants in solutions at higher PO_4 concentrations increased each week. The diameter of the plants increased with the increased phosphate concentrations of the culture solutions. Plants which were grown at 0.2 and 0.5 p.p.m. PO_4 were stocky, whereas those grown at 0.05 and 0.1 p.p.m. were spindling. The sorghum plants in the various solutions grew at a slower rate than the corn plants, otherwise the rate of growth was influenced by the PO_4 concentration of the culture solution in a similar way.

No measurements were recorded after the plants were 8 weeks old, but the rate of growth increased with the PO_4 concentration of the culture solution until harvest.

The rate of growth was also determined by weighing the plants each week, after allowing the solution to drain from the root system. The results are shown in table 9. There was practically no difference in the rate of corn growth in culture solutions with various phosphate concentrations during the

TABLE 9

Increase in green weight of 3 plants grown in culture solutions at PO_4 concentrations indicated

GROWTH PERIOD	CORN				SORGHUM				TOMATOES			
	PO_4 (p.p.m.) in culture solutions				PO_4 (p.p.m.) in culture solutions				PO_4(p.p.m.)in culture solutions			
	0.05	0.10	0.20	0.50	0.05	0.10	0.20	0.50	0.05	0.10	0.20	0.50
weeks	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
2	6.9	4.7	5.1	7.8	3.4	1.2	3.3	0.8	12.2	11.4	15	29
3	12.0	12.3	13.0	11.7	1.0	1.3	2.2	2.5	1.0	3.5	2.1	13
4	14.0	26.2	40.2	91.5	1.5	4.2	3.8	7.5	0.0	3.0	3.5	21
5	1.2	35.5	96.7	261.0	0.3	3.0	7.5	22.2	0.0	10.1	3.0	14
6	7.9	69.3	211.0	546.0	4.8	12.3	34.2	59.0	0.8	26.0	20.4	59
7	4.5	134.0	577.0	1,133.0	0.0	28.0	86.0	150.0	0.0	15.0	39.0	142
8	0.0	174.0	667.0	776.0	0.0	72.0	155.0	166.0	0.0	64.0	44.0	236

second week. But after this period the rate of growth increased markedly with the increased phosphate concentration, as measured by the weight of the plants. Corn grown at 0.05 p.p.m. PO_4 made practically no growth after the fourth week, whereas at the other PO_4 concentrations the plants grew rapidly each week. Sorghum grew much slower than corn, but the PO_4 in the culture solutions affected its growth in the same direction. It may be seen from table 9 that the tomato plants grew more rapidly in the solutions containing 0.5 p.p.m. PO_4 than did those in solutions at 0.1 and 0.2 p.p.m. PO_4. The average weekly increase at the highest PO_4 concentration was about 80 gm. compared to 20 gm. at 0.1 p.p.m. PO_4, whereas there was practically no growth at 0.05 p.p.m. PO_4.

After these plants were harvested, they were divided into three parts; leaves, stems, and roots. It was desirable to obtain a small quantity of sap from the leaves and stems. Therefore, it was necessary to determine the green weights

TABLE 10

Dry weight of plants grown in culture solutions of the phosphate content indicated

PO_4	CORN—6 PLANTS					SORGHUM—2 PLANTS					TOMATOES—3 PLANTS				
	Stems	Leaves	Tops	Roots	Total	Stems	Leaves	Tops	Roots	Total	Stems	Leaves	Tops	Roots	Total
p.p.m.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0.05	0.5	3.6	4.1	3.1	7.2	....*	....*	....*	0.3	.....*	....*	....*	.....*	0.3	.....*
0.10	78.1	62.5	140.6	45.7	186.3	18.7	9.5	28.2	14.7	42.9	17.0	17.8	34.8	19.1	53.9
0.20	663.9	139.0	802.9	98.6	901.5	48.4	13.0	61.4	18.1	79.5	22.7	30.9	53.6	20.8	74.4
0.50	946.0	179.8	1,125.8	136.8	1,262.6*	81.6	17.5	99.1	14.5	113.6	71.9	54.1	126.0	45.8	172.8
High-PO_4 Soil	118.0	86.1	204.1	123.0	327.1	59.0	6.6	65.6	13.3	78.9	....	....	.....	....	.....
Low-PO_4 Soil	90.6	84.0	174.6	51.0	225.6	25.8	6.5	32.3	7.2	39.5	....	....	.....	....	.....

* Not determined.

of the plants grown in the various cultures. After a representative sample of the green material for the sap studies was obtained, the remaining green material was dried in an oven for three days at 70°C.; the dry weights were determined and the total dry weights of the plants produced in each culture calculated. The average dry weight of the plants grown in duplicate cultures is given in table 10.

Corn made very little growth in the culture solution containing 0.05 p.p.m. PO_4; the dry weight of the tops was only 4.1 gm. The corn tops grown at 0.10 p.p.m. PO_4 made about 35 times as much growth as those grown at 0.05 p.p.m. whereas the roots were about 15 times as heavy as those at the lowest PO_4 concentration. The dry weights of the corn plants increased with the PO_4 concentration of the nutrient solution. A concentration of at least 0.50 p.p.m. PO_4 is required for maximum growth of corn. It is unfortunate that phosphate concentrations higher than 0.5 p.p.m. were not included in this study. The data indicate that high PO_4 concentration in the culture solutions increased the weight of the corn stems to a greater extent than that of the leaves. The top:root ratio increased with the phosphate concentration of the culture solution.

The low-PO_4 soil cultures, containing approximately 0.02 p.p.m. inorganic PO_4 in the soil solution, produced a better growth of corn than did the culture solutions with 0.1 p.p.m. PO_4. Corn also grew better in the high-PO_4 soil cultures than in culture solutions at 0.1 p.p.m. PO_4. The PO_4 content of the soil solution was not determined in this experiment, but it was probably not more than 0.1 p.p.m. PO_4.

The growth of sorghum and tomatoes increased with the phosphate concentration of the culture solution, as shown in table 10. The dry weights of plants grown at 0.05 p.p.m. PO_4 are not recorded because the plants were misplaced after the sap was obtained; they made, however, practically no growth in the culture solutions. The dry weight of sorghum tops was approximately 60 per cent more at 0.5 p.p.m. PO_4 than at 0.2, whereas the increase in the dry weight of tomato tops was 135 per cent. It was shown in experiment 1 that sorghum and tomatoes made about the same growth in culture solutions containing 0.2 and 0.5 p.p.m. PO_4. This difference may be due to the more favorable growing season. If a factor is limiting growth in some of the cultures and not in others, the differences in growth will be greater with more favorable temperature and light conditions.

PO_4 content of plants and plant sap

The dry tissue of the plants that were grown in culture solutions containing various phosphate concentrations was finely ground and the percentage of PO_4 determined by the method used in experiment 1. The average results of duplicate determinations are shown in table 11.

The data show that the PO_4 content of the plants increases with increasing concentrations of PO_4 in the culture solutions, just as in experiment 1. The

increase was greatest in corn and smallest in sorghum. With all of the crops the increase in PO_4 content was greater in the tops than in the roots. The roots of the plants contained a higher percentage of PO_4 than the leaves when grown at 0.05 and 0.1 p.p.m. PO_4, but the reverse is true of the plants grown at 0.2 and 0.5 p.p.m. PO_4. Tomatoes had a higher percentage of PO_4 than corn or sorghum. The percentage of PO_4 in the plants grown in experiment 2 is lower than that of those grown in experiment 1.

The sap from the plants grown in culture solutions with different PO_4 concentrations was obtained and clarified; and the inorganic PO_4 content was determined as described in experiment 1. The results are recorded in table 12.

TABLE 11

PO_4 content of dry tissue of plants grown in culture solutions of the phosphate content indicated

PO_4 IN CULTURE SOLUTION	CORN			SORGHUM			TOMATOES		
	Leaves	Stems	Roots	Leaves	Stems	Roots	Leaves	Stems	Roots
p.p.m.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.05	0.25	0.15	0.44	....	0.11	0.46	0.39	No determi-	1.17
0.10	0.38	0.27	0.40	0.33	0.29	0.42	0.81	nations	0.85
0.20	0.60	0.43	0.46	0.41	0.35	0.53	0.75	made	0.96
0.50	0.82	0.62	0.73	0.63	0.58	0.63	1.14		1.34

TABLE 12

PO_4 content of sap from plants grown in culture solutions with phosphate concentrations indicated

PO_4 IN CULTURE SOLUTION	CORN		SORGHUM		TOMATOES	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
0.05	380	47	...	..	...	...
0.10	235	55	215	52	210	111
0.20	490	51	385	58	315	161
0.50	1,250	141	581	82	438	500

The phosphate concentration of the culture solutions influenced the PO_4 content of the plant sap. The data show that the sap from the leaves contains a higher concentration of PO_4 than that fron the stems except in the case of tomatoes grown at 0.5 p.p.m. PO_4. Sap from corn plants grown at 0.05 p.p.m. PO_4 contained a rather high PO_4 content, but this may be attributed to the fact that the plants were dried and practically dead when harvested. Sap from corn leaves grown at 0.1 p.p.m. PO_4 contained about 235 p.p.m. PO_4 whereas the sap from those grown at 0.5 p.p.m. PO_4 contained approximately 1,250 p.p.m.

The sap from sorghum leaves and stems contained less PO_4 than that from corn leaves and stems. No sap could be obtained from sorghum grown in culture solutions at 0.05 p.p.m. PO_4 because the plants were dead when

harvested. An increased PO_4 concentration in the culture solution increased the PO_4 content of the sap from sorghum and tomato plants just as in the case of corn. The sap from tomato leaves had a lower PO_4 content than that from corn and sorghum.

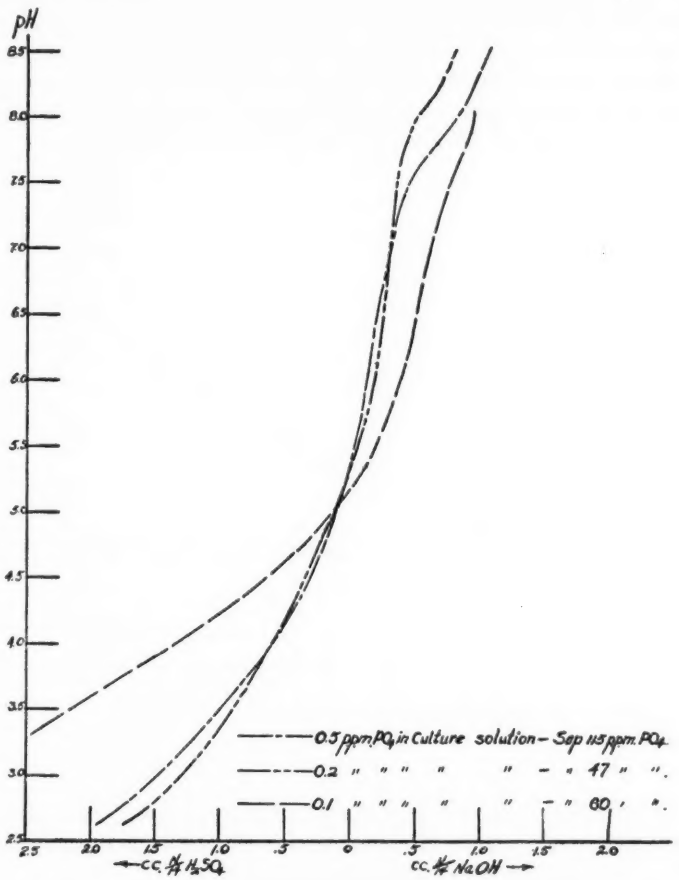

FIG. 1. PHOSPHATE CONTENT AND TITRATION CURVES FOR SAP FROM CORN STEMS GROWN WITH DIFFERENT CONCENTRATIONS OF PHOSPHATE

Buffer capacity of plant sap as related to its PO_4-content

As shown in experiment 1, the PO_4 content of the plant sap was high even in plants that did not make maximum growth. The question arises as to why such concentrations of PO_4 in the sap are inadequate for maximum growth. If

even higher concentrations of PO_4 in the plant sap are necessary for maximum growth, what is the function of the phosphate in the sap? It seemed possible that the phosphate played an important rôle as buffer material in the plant sap and that the high concentrations were essential for properly buffering the sap. Such an assumption seemed to be in accord with the results obtained by Martin (6) on the relation between the PO_4 content and buffer capacity of sap from the leaves and stems of the sunflowers. She found that the buffer capacity of the plant sap was always equal to that which should have been caused by the PO_4 contained in it. She concluded, therefore, that phosphate is the principal buffer in the plant. If her conclusion is correct, the plant sap

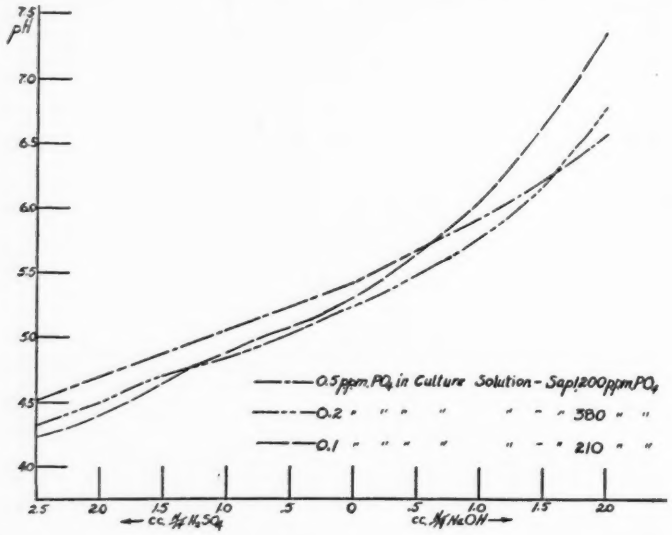

FIG. 2. PHOSPHATE CONTENT AND TITRATION CURVES FOR SAP FROM CORN LEAVES GROWN WITH DIFFERENT CONCENTRATIONS OF PHOSPHATE

containing 1,200 p.p.m. PO_4 should be much better buffered than that containing 200 p.p.m. In order to study this question, the buffer capacity of sap from plants grown in experiment 2 was determined.

In making the study of buffer capacity, the sap was obtained as previously indicated. Its buffer capacity was determined by the change in reaction caused by small additions of $N/14$ H_2SO_4 and NaOH to 5 cc. of the sap. The reaction was determined by means of the quinhydrone electrode.

The titration curves are shown in figures 1 to 4, inclusive. Figure 1 shows the PO_4 content and the titration curves for the sap from corn stems grown in culture solutions with 0.1, 0.2, and 0.5 p.p.m. PO_4. There is no difference in the buffer capacity of sap which contained 47 p.p.m. and that which con-

tained 115 p.p.m. PO_4. The sap with 60 p.p.m. PO_4 had a greater buffer capacity than that containing more PO_4. Figure 2 shows the PO_4 content and the titration curves for the sap from corn leaves grown in culture solutions at various PO_4 concentrations. These curves indicate very little difference in the buffer capacity of sap containing 210, 380, or 1,200 p.p.m. PO_4. The PO_4 content of the sap from sorghum stems did not affect its buffer capacity as is indicated in figure 3. There is no difference in the buffer capacity of tomato stem sap which contained 500 p.p.m. and that which contained 161 p.p.m. PO_4 (fig. 4). The buffer capacity of the sap which contained 111

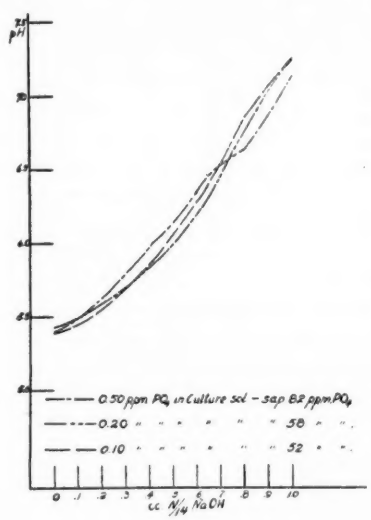

FIG. 3

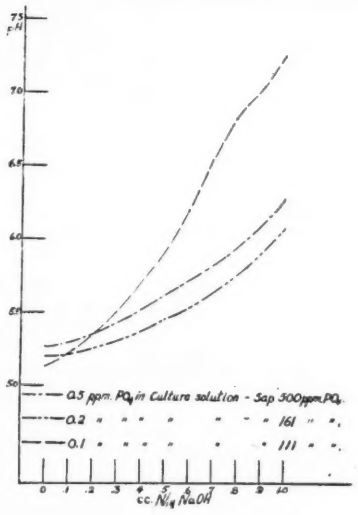

FIG. 4

FIG. 3. PHOSPHATE CONTENT AND TITRATION CURVES FOR SORGHUM STEMS GROWN WITH DIFFERENT CONCENTRATIONS OF PHOSPHATE

FIG. 4. PHOSPHATE CONTENT AND TITRATION CURVES FOR TOMATO STEMS GROWN WITH DIFFERENT CONCENTRATIONS OF PHOSPHATE

p.p.m. PO_4 is much less than that with a higher PO_4 content. In a few cases, there seems to be a rather marked displacement of the curve and perhaps an increased H-ion concentration with low PO_4 content. Probably this is due to the influence of phosphate on metabolic processes concerned with the formation of organic acids, rather than to a lack of phosphate for a buffer material.

The results given in the foregoing indicate very clearly that phosphates play a minor rôle as buffer material in the plant sap and that the apparent necessity of a high phosphate content of plant sap cannot be explained on the basis of its functioning as buffer material.

EXPERIMENT 3

This experiment was planned to study the influence of the reaction of the culture solution on the rate of phosphate absorption by plants. Theron (13) found that the reaction of the culture solution had an appreciable effect on the absorption of Ca, Mg, K, and NO_3 ions. The cations were absorbed more rapidly from an alkaline solution than from an acid solution by barley and

TABLE 13

PO_4 absorbed in 3 days by 16 corn plants at reactions indicated

TRIAL	PO_4 ABSORBED FROM CULTURE SOLUTIONS OF			
	pH 4	pH 5	pH 6	pH 7.5
	mgm.	*mgm.*	*mgm.*	*mgm.*
1	214	204	138	15
2	164	140	108	44
3	305	266	220	40
Average.....	227	203	155	33

TABLE 14

PO_4 absorbed by 36 wheat plants at reactions indicated

TRIAL	ABSORPTION PERIOD	PO_4 ABSORBED FROM CULTURE SOLUTIONS OF			
		pH 4	pH 5	pH 6	pH 7.5
	hours	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	3	36	36	44	8
2	3	20	18	28	0
3	10	162	208	202	84
4	10	192	202	194	56
5	10	126	110	160	4
6	10	148	146	174	8
7	24	271	263	266	134
8	24	265	274	267	104
9	24	277	282	287	222
10	24	263	269	270	152
Average.....	..	176	180	185	77

cucumbers. The NO_3 ions were absorbed more rapidly from acid solutions whereas the absorption of PO_4-ions was very irregular. A more detailed study of the influence of the reaction of the culture solution on the absorption of PO_4 was thought desirable.

In an experiment of this kind uniform plants are very essential. Therefore, a large number of seeds were germinated in quartz sand so that a very careful selection of seedlings could be made. Only those having the same appearance, height, and root development were used. Four corn seedlings were placed

in each 100-liter culture vessel. The composition of the culture solution is given in table 1. These plants were grown in the culture solution at the same reaction (pH 6.5) with a very limited amount of phosphate for three weeks. At the end of this period, the plants appeared uniform as to top and root growth, but it was desirable to find out whether or not they were uniform as to PO_4 absorption. The PO_4 content of the culture solutions was adjusted to 1 p.p.m. each day, and the amount of PO_4 absorbed by the plants in each culture was determined for three successive days. In most of the cases the plants in each culture absorbed practically the same amount of phosphate, and those which were not uniform in this respect were discarded. The culture solution were then adjusted to the desired reactions; pH 4, 5, 6, and 7.5 and these reactions were maintained by frequent additions of H_2SO_4 or NH_4OH. There were 4 cultures, or 16 corn plants, and 3 cultures, or 36 wheat plants, at each reaction. The PO_4 content of the culture solutions was again adjusted to 1 p.p.m. each day by the addition of potassium acid phosphate.

The amount of PO_4 absorbed from the culture solutions with the various reactions was determined for several 3-day periods. After each absorption period the plants were placed in 4-gallon pots of tap water for a few days before another absorption period. During the second and subsequent absorption periods the plants were interchanged so that they were never placed in the same solution during any two periods. That is, the plants which were in a solution having a reaction of pH 4 during the first absorption period were subsequently placed in solutions having reactions of pH 5, 6, and 7.5.

The results are shown in tables 13 and 14. The PO_4 absorption by corn (table 13) increased with the acidity of the culture solutions. The PO_4 absorbed was about 30 to 40 per cent more at pH 5 than at 6. In every case the absorption was less at pH 7.5 than at any other reaction. There was no difference in the PO_4 absorption by the wheat plants from the acid culture solutions, as shown in table 14, but the PO_4 absorption was less from the culture solutions having a reaction of pH 7.5.

DISCUSSION

The results obtained in experiment 1 show that a phosphate concentration of at least 0.5 p.p.m. is required for the maximum growth of corn. It also indicates that the maximum growth of tomatoes and sorghum may be obtained at a phosphate concentration of 0.2 p.p.m. The results obtained in experiment 2 are in general agreement with those of experiment 1, except that a concentration of 0.5 p.p.m. PO_4 was required for maximum growth. The cause for this difference is not known, but it may be climatic factors. These results differ considerably from those obtained by Parker and Pierre (11), in that those investigators obtained the maximum growth of corn at a phosphate concentration of 0.25 p.p.m. The cause of this failure to agree with their results is not evident, for the general procedure used in the two investigations was similar, the only known difference being the addition of boron to the culture solution

in the recent experiments. This seems unimportant, however, for Parker and Pierre used tap water and commercial chemically pure salts in preparing their solutions.

When the relation to data on the PO_4 content of the soil solutions is considered, the data obtained in this investigation show rather conclusively the inadequacy of the soil solution, as obtained by displacement, for the growth of plants. Parker (8) has discussed this relation rather fully, so a detailed consideration of it will not be given at this time. In this investigation, however, additional data bearing on the point were obtained by including a few soil cultures for comparison with the solution cultures. As was indicated in experiment 2, corn growing in a soil whose displaced solution contained only 0.02 or 0.03 p.p.m. inorganic PO_4 made better growth than corn in a culture solution containing 0.1 p.p.m. PO_4.

It is apparent from the foregoing that the root-soil contact is essential for good plant growth in some soils. Comber (2), Parker (8), Hoagland (5), and Truog (14) have discussed this phase of the subject in detail. It will not be discussed here other than to emphasize the fact that it is essential, if we are to account for the absorption of phosphate from many soils of the humid region.

The studies on the composition of the plant and plant sap indicate, as expected, that their phosphate content increases with an increase in the concentration of the culture solution. The sap from the plants which made maximum growth had a very high phosphate content. For maximum growth, why was it necessary for the sap to contain such high PO_4 concentrations? No attempt to answer this question is made, further than to show that the buffer capacity of the sap is not influenced by its phosphate content.

SUMMARY

Two experiments are given in which a study was made of the concentration of PO_4 required for the maximum growth of corn, sorghum, and tomatoes. In addition, the influence of the PO_4 concentration of the culture solution on PO_4 absorption, on rate of growth, on percentage of PO_4 in the dry tissue, on PO_4 content of the plant sap, and on buffer capacity of the sap was studied. The experimental procedure was described in detail. A comparison of the growth of corn and sorghum in soil and solution cultures was made. The influence of the reaction of the culture solution on the rate of PO_4 absorption was studied. The growth of plants in soil and solution cultures was briefly discussed. The results of this investigation may be summarized as follows:

1. Culture solutions of low phosphate concentrations were maintained very well by the use of a large volume of solution for each plant and by the addition of potassium acid phosphate at frequent intervals.
2. The rate of PO_4 absorption was not directly proportional to the PO_4 concentration of the culture solution.
3. The rate of growth increased with increasing phosphate concentrations throughout the growing period.

4. Maximum growth of corn, sorghum, and tomatoes was obtained at 0.5 p.p.m. PO_4. Growth of corn and sorghum at 0.2 p.p.m. PO_4 was good, the dry weight being about 71 per cent of the maximum. The dry weight of tomatoes at 0.2 p.p.m. was only 42 per cent of the maximum.

5. Plants made a better growth in soil whose displaced solution contained from 0.02 to 0.03 p.p.m. inorganic PO_4 than in culture solutions at 0.1 p.p.m. PO_4.

6. The phosphate content of plant and plant sap increased with increasing concentrations of PO_4 in the culture solutions. Tomatoes had a higher percentage of PO_4 than corn or sorghum, whereas the sap from corn and sorghum leaves had a PO_4 content higher than that of tomatoes.

7. Phosphate played a minor rôle as a buffer material in the plant sap.

8. Corn and wheat plants absorbed PO_4 more rapidly from acid than from alkaline culture solutions. There was no appreciable difference in the rate of PO_4 absorption from culture solutions having a reaction of pH 4, 5, and 6.

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AN IMPROVED METHOD FOR THE DETERMINATION OF AVAILABLE PHOSPHORIC ACID OF SOILS

SURENDRALAL DAS

Agricultural Research Institute, Pusa, India

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In a previous publication (3) the author has shown that Dyer's citric acid method breaks down as a discriminating agent for evaluating the available phosphoric acid of calcareous soils, and that 1 per cent potassium carbonate solution is capable of differentiating plots of known cropping and manurial history and thus gives an indication of the probable fertility of calcareous soils in their relation to available phosphoric acid. The present investigation was undertaken to determine whether this new method could be applied to other types of soil with equal advantage and thus rendered a universal method for the determination of available phosphoric acid of soils in general.

It is generally conceded that plant-food exists in soils in two forms: one is often referred to as easily soluble, active or available, and consists of that smaller portion which can be utilized by plants for their immediate needs as distinguished from the second form, the larger but less available reserve supply in the soil.

Numerous procedures have been adopted for the estimation of the easily soluble plant-food in soils. The most popular is the weak acid digestion. The mineral acids, hydrochloric or nitric acid, are employed in the United States, whereas citric acid has found favor in Europe and elsewhere.

The reliability of these laboratory methods has been questioned from more than one quarter, and, no doubt, on account of the difficulty of reproducing soil conditions in the laboratory, they have always been justly considered more or less arbitrary. However, it may be safely stated that some of them do give results of a certain value when properly interpreted, as their findings have often been verified in the field; but it is doubtful whether in the present state of our knowledge we can evolve a method on which absolute reliance could be placed.

In this connection the work done at the Texas Agricultural Experiment Station (6, 10, 11) may be of interest. There the solubility of soil minerals has been studied by bringing phosphate or potash minerals into contact with 0.2 *N* HNO_3—used as a substitute for 1 per cent citric acid solution—in the proportions in which these minerals may occur in the soil and under the conditions of the extraction. Calcium phosphate and precipitated phosphates of iron and aluminium are completely soluble, and vivianite and triplite are nearly so in 0.2 *N* HNO_3. The aluminum phosphates, e.g., variscite and wavellite, and the basic ferric phosphates are comparatively slightly soluble.

Ferrous phosphate, vivianite, rarely exists in ordinary cultivated soils, although it may be found in some soils suffering from lack of aeration. As 0.2 *N* HNO_3 dissolves calcium phosphates completely, but mineral aluminum or basic ferric phosphates to only a slight extent, it differentiates between these two classes of phosphate compounds in the soil. Apatite, phosphate rock, precipitated ferric and aluminum phosphates, vivianite, and triplite are almost equally soluble. Acid phosphate may also be taken as completely soluble. But these compounds can hardly be claimed to possess the same value to plants, nor is a solvent now known which would dissolve phosphoric acid from the phosphates mentioned, in the same proportions as it would be assimilated from them by plants. What we cannot do with known mineral phosphates of known composition and character outside of the soil, we can hardly expect to do with the same phosphates when they are incorporated in the soil, and far less with the unknown phosphates already locked up within the soil.

Soils may, therefore, contain equal quantities of phosphoric acid soluble in 0.2 *N* HNO_3 and yet liberate unequal quantities for plants, on account of differences in the phosphates present. This consideration must give rise to caution while different types of soil are being compared with one another. Only those soils should be compared which probably contain the same kinds of phosphates. Soils widely dissimilar in origin and character can hardly be compared, unless there is strong evidence that they contain similar phosphates.

Moreover, the nature of the plant which is grown, apart from other factors, must always play an important rôle with regard to the avialability of different phosphates present in soils or applied to the same as fertilizers. Truog (9), in a comparison of 10 different kinds of plants grown under greenhouse conditions with acid phosphate, rock phosphate, precipitated calcium phosphates, aluminum phosphate, iron phosphates (both ferrous and ferric), magnesium phosphate, or manganese phosphate as sources of phosphoric acid, found that, contrary to the general belief in the relative unavilability of aluminum and iron phosphates to plants, 9 out of 10 plants made better growth on alumnium phosphate than on calcium phosphates, and 6 made better growth on ferric phosphate. This clearly indicates the inadequacy of chemical solvents in measuring the availability of different phosphates.

Further, many investigators (2,10,11) have observed that the growing plant itself possesses more or less power to feed directly on phosphates and that some plants possesss specially marked powers. As a result, no common limiting figure for available phosphoric acid can be suggested, as given by Dyer (4), which will be equally applicable to all types of soil, for the figure varies not only with the character of the soil, but also depends on the kinds of crops grown. Consequently, this figure must be worked out for different soils, and it will vary even in the same soil according to the type of crop grown on it from time to time.

Fraps (5) has dealt with this problem of availability of plant-food in soils from a different stand-point. According to his findings the amount of any

given plant-food which is withdrawn from the soil by the plant depends upon a number of factors, which are classified as follows.

1. The amount of plant-food present at the beginning of the growing season in a form which can be partly or completely absorbed by the plant is termed *chemically available.*

2. Compounds chemically available may be enclosed within the soil particles so as not to be exposed to the action of plant roots. Such compounds are *physically unavailable.* If the encrusting substance is removed, such compounds become chemically available.

3. The amount of plant-food transformed during the growing season into forms of combination which can be absorbed by plants may be measured in terms of *weathering availability.* This factor is certainly of importance with respect to nitrogen. Its importance in the case of potash and phosphoric acid is apparently not so great; but the matter requires study.

4. Plants differ both in their capacity to absorb food and in their need of it. Whatever may be the cause of these differences, there is no doubt that they exist. This factor is designated *physiological availability.*

The character of the soil, its chemical composition, the conditions which prevail during the growth of the plant, the incidence of sunlight, the effective range of temperature, the nature of the microflora present, and the movement of air and water must bring into play several factors affecting the availability of plant-food in soils. Moreover, our knowledge of soil physics and plant physiology needs further development before we can positively assert much in this direction. Physico-chemical studies, however, relating to base exchange and colloidal behavior are likely to give the soil chemist a powerful weapon to combat hitherto baffling problems and to throw new light on many unexplained soil phenomena.

The extraction with 1 per cent potassium carbonate solution has proved useful for the estimation of available phosphoric acid of calcareous soils (3). The underlying principle of its action on calcareous soils is twofold: a reaction takes place with any dicalcic or such other phosphates present in these soils, with the production of insoluble tricalcic or other phosphates and of soluble potassium phosphate; and, further, phosphorus in organic combination in humus is also dissolved.

Dyer's 1 per cent citric acid solution has been shown to be unsuitable for calcareous soils (3), and therefore the results obtained with other types of soil by this method cannot be compared with those of calcareous soils. On the other hand, if the extraction with 1 per cent potassium carbonate solution which has been found useful for calcareous soils, proves equally effective for other types of soil too, then it can be recognized as a more or less universal method for the estimation of available phosphoric acid of soils in general.

In order to test this point as well as to compare the two methods, many typical soils from different parts of India were collected for examination, the cropping and manurial history of these soils being accurately known. In a few cases the total phosphoric acid of soils was also determined with a view to finding out whether it had any significant relationship to available phosphoric acid. The method of extraction with 1 per cent potassium carbonate solution has already been described (3) and is practically the same as Dyer's

TABLE 1

A comparison of total and available phosphoric acid by citric acid and potassium carbonate methods in acid soils of known cultural history

DESCRIPTION OF PLOTS	CROPS GROWN	PER CENT TOTAL P_2O_5 (T)	PER CENT AVAILABLE P_2O_5		$\frac{K}{T}$	$\frac{C}{T}$
			By citric acid (C)	By K_2CO_3 (K)		
Tocklai Indian Tea Association, Assam						
Borbhetta-Kharikatia Plots:						
No manure	Tea	0.0352	0.0023	0.0080	0.228	0.066
Superphosphate	Tea	0.0414	0.0051	0.0112	0.270	0.124
Basic slag	Tea	0.0454	0.0057	0.0105	0.230	0.126
Limestone	Tea	0.0406	0.0019	0.0064	0.157	0.046
Limestone + K_2SO_4	Tea	0.0432	0.0053	0.0131	0.304	0.123
Limestone + superphosphate	Tea	0.0436	0.0050	0.0103	0.237	0.114
K_2SO_4	Tea	0.0317	0.0020	0.0073	0.231	0.063
K_2SO_4 + superphosphate	Tea	0.0443	0.0049	0.0129	0.292	0.112
Borbhetta-Betjan Plots:						
No manure	Tea	0.0413	0.0027	0.0099	0.240	0.066
Manured	Tea	0.0450	0.0041	0.0126	0.281	0.092
Borbhetta-Matelli Plots:						
Bonedust	Tea	0.0434	0.0039	0.0079	0.183	0.090
Bonedust + fish guano	Tea	0.0429	0.0042	0.0091	0.263	0.098
Bonedust + nitrolim	Tea	0.0484	0.0034	0.0065	0.134	0.071
Bonedust	Tea	0.0405	0.0045	0.0107	0.263	0.110
Bonedust + $NaNO_3$	Tea	0.0407	0.0039	0.0080	0.197	0.096
Bonedust + $(NH_4)_2SO_4$	Tea	0.0421	0.0040	0.0080	0.208	0.094
Bonedust + mustard cake	Tea	0.0431	0.0041	0.0087	0.201	0.096
Block E, Jorhat Government Farm, Assam						
Non-phosphated	Sugar cane (*S. Mauritus*)	.....	0.0034	0.0083	.....	.....
Phosphated	Sugar cane (*S. Mauritus*)	.....	0.0043	0.0093	.....	.....
Non-phosphated	Sugarcane, D. 74	.....	0.0038	0.0090	.....	.....
Phosphated	Sugarcane, D. 74	.....	0.0066	0.0096	.....	.....
Block D, Jorhat Government Farm, Assam						
Non-phosphated	Sugarcane, D. 74	.....	0.0019	0.0079	.....	.....
Phosphated	Sugarcane, D. 74	.....	0.0017	0.0113	.....	.....
Block E, Karimganj Government Farm, Assam						
Control	Paddy	.....	0.0006	0.0062	.....	.....
Bonemeal	Paddy	.....	0.0021	0.0074	.....	.....

TABLE 1—*Concluded*

DESCRIPTION OF PLOTS	CROPS GROWN	PER CENT TOTAL P_2O_5 (T)	PER CENT AVAILABLE P_2O_5		$\frac{K}{T}$	$\frac{C}{T}$
			By citric acid (C)	By K_2CO_3 (K)		
Hmwabi Experimental Station, Burma						
Bad, unmanured	Paddy	0.0339	0.0021	0.0025	0.074	0.061
Good, unmanured	Paddy	0.1109	0.0444	0.0260	0.235	0.400
No manure	Paddy	0.0409	0.0032	0.0037	0.090	0.078
Bonemeal	Paddy	0.0520	0.0097	0.0060	0.116	0.186
Cinchona Camp, via Margui, South Burma						
Bad soil from ridge	Cinchona	0.0350	0.0015	0.0046	0.131	0.043
Good soil from slope	Cinchona	0.1720	0.0090	0.0079	0.046	0.052

citric acid method except for certain details. For easy reference, however, it is briefly stated in the following.

One-hundred grams of air-dry soil passing 2-mm. mesh are shaken with 1 liter of 1 per cent potassium carbonate solution for 24 hours in a mechanical shaker. The extract, containing some potassium carbonate in the free state, is separated by suction and neutralized almost wholly with nitric acid and then with a little hydrochloric acid; as a result a considerable amount of potassium nitrate is produced in the solution, which helps the granular precipitation of ammonium-phosphomolybdate in the ammonium molybdate method of estimating phosphoric acid.

Soils collected may be broadly divided into three groups; namely,

1. *Acid soils*, in which are also included *humus* and *laterite* soils.
2. *Alkali soils*, which also include *calcareous* soils.
3. *Non-calcareous soils*, including various typical soils not belonging to 1 and 2.

ACID SOILS, INCLUDING HUMUS AND LATERITE SOILS

Acid soils were obtained from the Government Farms of Jorhat and Karimganj, the Tocklai Experimental Station of the Indian Tea Association, Assam, and also from Burma. The soils were collected from plots whose cropping and manurial history was known. The pH of these soils varies from 3.0 to 6.0. The results obtained are set forth in table 1.

It will be noticed that the analytical figures for phosphoric acid obtained with 1 per cent potassium carbonate extraction serve to differentiate manured from unmanured plots, and compare well with those given by Dyer's citric acid method. In some cases total phosphoric acid was determined in order to see whether it had any relationship with the values of available phosphoric acid. The ratio of K_2CO_3-soluble to total phosphoric acid varies from 0.13 to 0.30 in the case of acid soils of Tocklai Indian Tea Association, Assam,

TABLE 2

Comparison of total and available phosphoric acid by citric acid and potassium carbonate methods in laterite soils of known cultural history

DESCRIPTION OF PLOTS	CROPS GROWN	PER CENT TOTAL P_2O_5 (T)	PER CENT AVAILABLE P_2O_5		$\frac{K}{T}$	$\frac{C}{T}$
			By citric acid (C)	By K_2CO_3 (K)		
East Suti Plots, Dacca						
No manure	Paddy and maize	.....	0.0013	0.0079	.....	.....
Bonemeal	Paddy and maize	.....	0.0017	0.0097	.....	.....
Basu's Experimental Plots, Dacca						
Bonemeal	Paddy	.....	0.0020	0.0081	.....	.....
Bonemeal + lime	Paddy	.....	0.0015	0.0063	.....	.....
North Hazi Plots, Blocks B and C, Dacca						
No manure	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0618	0.0021	0.0075	0.121	0.034
Bonemeal	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0854	0.0067	0.0099	0.115	0.078
Lime	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0675	0.0043	0.0065	0.096	0.064
Lime + bonemeal	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0827	0.0127	0.0081	0.098	0.154
Cowdung	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0639	0.0025	0.0081	0.127	0.039
Cowdung + bonemeal	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0737	0.0089	0.0116	0.157	0.121
Cowdung + lime	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0596	0.0027	0.0065	0.109	0.045
Cowdung + lime + bonemeal	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0891	0.0154	0.0100	0.112	0.173

TABLE 2—*Concluded*

DESCRIPTION OF PLOTS	CROPS GROWN	PER CENT TOTAL P_2O_5 (T)	PER CENT AVAILABLE P_2O_5		$\frac{K}{T}$	$\frac{C}{T}$
			By citric acid (C)	By K_2CO_3 (K)		
North Hazi Plots, Blocks A and D, Dacca						
Green manure	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0609	0.0018	0.0072	0.118	0.030
Green manure + bonemeal	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0722	0.0042	0.0090	0.125	0.058
Green manure + cowdung	Mustard, paddy, and matikalai (*Phaseolus mungo*)	.....	0.0021	0.0096	.....	.....
Green manure + cowdung + lime	Mustard, paddy, and matikalai (*Phaseolus mungo*)	.....	0.0007	0.0075	.....	.....
Green manure + lime	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0489	nil	0.0054	0.110	.....
Green manure + lime + bonemeal	Mustard, paddy, and matikalai (*Phaseolus mungo*)	0.0833	0.0079	0.0071	0.085	0.095
Green manure + bonemeal + cowdung	Mustard, paddy, and matikalai *Phaseolus*) *mungo*)		0.0059	9.0128	.....	.....
Green manure + bonemeal + cowdung + lime	Mustard, paddy, and matikalai (*Phaseolus mungo*)	....	0.0128	0.0100	.....	.....

whereas the corresponding ratio in the case of citric-soluble phosphoric acid ranges between 0.04 and 0.12. The variation of these ratios is still greater in Burma acid soils. It will be evident that the greater fertility of the plots examined with respect to available phosphoric acid is usually accompanied by proportionally increasing ratios by both the methods.

Similarly, Lemmermann (7) and Andre and Copaux (1), from a determina-

tion of the ratio of citric-soluble to total phosphoric acid of soils of known agricultural history, concluded that the greater the ratio between citric-soluble and total phosphate, the smaller the return which follows the application of soluble phosphate, and that it is not so much the absolute dose of phosphoric acid that matters with regard to the factor of fertility as the size of the preceding ratio. Vanstone (12) also compares the value of determinations of this ratio with the information given by the Dyer's method.

Next, laterite soils collected from Dacca Central Farm were examined for available phosphoric acid. These soils are acid in reaction, the pH varying from 4.0 to 6.0. The results are given in table 2.

TABLE 3

Comparison of total and available phosphoric acid by citric acid and potassium carbonate methods in humus soils of known cultural history

DESCRIPTION OF PLOTS	CROPS GROWN	PER CENT TOTAL P_2O_5 (T)	PER CENT AVAILABLE P_2O_5		$\frac{K}{T}$	$\frac{C}{T}$
			By citric acid (C)	By K_2CO_3 (K)		
Hill soils, Simla						
Polo ground	Grassland	0.286	0.0622	0.0348	0.122	0.218
Race course	Grassland	0.360	0.1208	0.0424	0.118	0.336
Somerford Orchard, Ramgarh, District Nainital, U. P.						
Soil no. 2	Fruit trees	0.136	0.0131	0.0106	0.078	0.097
Soil no. 3	Fruit trees	0.235	0.0409	0.0095	0.040	0.174
Hill soils, Cloud End, Mussoorie						
Soil no. A	Fruit trees	0.159	0.035	0.0104	0.065	0.220
Soil no. C	Fruit trees	0.377	0.117	0.0348	0.092	0.470
Soil no. D	Fruit trees	0.327	0.048	0.0216	0.066	0.147
Soil no. E	Fruit trees	0.353	0.100	0.0322	0.091	0.283
Soil no. F	Fruit trees	0.195	0.015	0.0148	0.076	0.077

The same remarks apply here as in the case of acid soils reported in table 1.

Hill soils are generally very rich in humus and available plant-food, but poor in lime content. They are acid in reaction. Some such soils of known agricultural history were obtained from Simla, Mussoorie, and Ramgarh for examination. The results are given in table 3.

Here, too, the same remarks apply as in the case of other acid soils of this group.

ALKALI SOILS INCLUDING CALCAREOUS SOILS

Results of alkali soils obtained from Sukkur Agricultural Station, Sindh, are given in table 4. Good soils represent those which are being reclaimed

under the Sukkur Barrage Scheme recently brought into being, and are more fertile than bad soils which have as yet been little reclaimed. The determination of total phosphoric acid was omitted in soils of this group.

It will be noticed that the citric acid method breaks down altogether as a criterion for distinguishing good soils from bad ones, and, as a matter of fact, carries an erroneous impression with regard to the fertility of these soils. On the other hand, the values obtained with potassium carbonate solution are borne out by the cultural history of the plots under examination.

In this group are also included calcareous soils obtained from the Bombay Presidency, Central Provinces, and Berars. The results are set forth in table 5.

In the case of soils from Manjri Dry Farming Station, Bombay, the citric acid extraction did not yield any phosphoric acid which could be estimated by ordinary methods of analysis. The figures obtained with potassium car-

TABLE 4

Comparison of available phosphoric acid by citric acid and potassium carbonate methods in alkali soils of known cultural history

DESCRIPTION OF PLOTS	CROPS GROWN	PER CENT AVAILABLE P_2O_5	
		By citric acid	By K_2CO_3
Bad soil	Sorghum, berseem and wheat	0.0198	0.0043
Good soil	Sorghum, berseem and wheat	0.0031	0.0071
Bad soil	Sorghum	0.0326	0.0036
Good soil	Sorghum	0.0206	0.0069

bonate solution, though not very conclusive, yet give some indication of the fertility of the lands. In the case of soils of Poona Agricultural College Farm, black soils of Nagpur College Farm, and black cotton soils of Akola Government Farm the potassium carbonate method gives values of available phosphoric acid which are clearly corroborated by field tests, whereas the results with the citric acid process are rather erratic.

Thus it will be noticed that in practically all cases examined, the method of extracting with 1 per cent potassium carbonate solution has differentiated between manured and unmanured plots and also between plots treated with phosphatic fertilizers and those treated with fertilizers containing no phosphates, whereas the citric acid method yields uneven and even misleading values in most cases.

In the present instance, as the total amount of phosphoric acid of these soils was not determined, the ratios of potassium carbonate-soluble or citric-soluble to total phosphate could not be compared. The results of calcareous soils detailed in a previous publication by the author (3), where total phosphoric

acid was determined, show that the greater fertility of soils under examination with respect to their available phosphoric acid is generally accompanied by proportionally bigger ratios obtained with the potassium carbonate method.

TABLE 5

Comparison of available phosphoric acid by citric acid and potassium carbonate methods in calcareous soils of known cultural history

DESCRIPTION OF PLOTS	CROPS GROWN	PER CENT AVAILABLE P_2O_5	
		By citric acid	By K_2CO_3
Manjri Dry Farming Station, Bombay			
Plot no. 2, control	Bajri (*Pennisetum typhoideum*)	nil	0.0013
Plot no. 6, farmyard manure	Bajri (*Pennisetum typhoideum*)	nil	0.0014
Plot no. 7, green manure	Bajri (*Pennisetum typhoideum*)	nil	0.0012
Control, shallow	Bajri (*Pennisetum typhoideum*)	nil	0.0011
Poona Agricultural College Farm, Bombay			
No manure	Horse gram	0.0024	0.0023
Plot no. 6A, formyard manure	Horse gram	0.0024	0.0036
Plot no. 6B and 5B, farmyard manure	Horse gram	0.0011	0.0034
Plot no. 23A, farmyard manure	Horse gram	0.0286	0.0050
Plot no. 21A and 22A, farmyard manure	Horse gram	0.0406	0.0065
Black soil, Nagpur College Farm, C. P.			
Dung + urine	Sorghum and gram*	0.0009	0.0012
Dry excreta	Sorghum and gram*	0.0002	0.0011
Urine	Sorghum and gram*	0.0010	0.0008
No manure	Sorghum and gram*	0.0016	0.0005
Scarified	Wheat†	0.0109	0.0023
Country ploughed and scarified	Wheat†	0.0076	0.0024
Inversion ploughed and scarified	Wheat†	0.0150	0.0032
Black cotton soils, Akola Government Farm, Berars			
Castor cake + super	Cotton*	0.0041	0.0011
Castor cake + super + lime	Cotton*	0.0006	0.0009
Castor cake + super + lime	Cotton*	0.0006	0.0006

* Yields in descending order.

† Yields in ascending order.

Such relationship would not obviously hold in the case of the Dyer's method, which gives rather erratic results in these soils.

NON-CALCAREOUS SOILS

These soils represent approximately neutral soils, being neither too acid nor too alkaline in reaction. The results are set forth in table 6. In a few cases total phosphoric acid was also determined.

It will be noticed that in practically all cases examined both the methods of extraction have differentiated between manured and unmanured plots, and also between plots treated with phosphatic fertilizers and those treated with other fertilizers containing no phosphates. It is only in the two groups of acid and non-calcareous soils that both methods agree closely, whereas in the case of calcareous and alkali soils the citric acid method gives erratic results. On the other hand, the method of extracting with 1 per cent potassium carbonate solvent gives, in a uniform manner and in all types of soil, values for available phosphoric acid which are significantly related to the cultural history of the plots, and are therefore comparable with regard to the probable fertility of soils in their relation to available phosphoric acid. This possibility is rather remote in the case of the citric acid process, for reasons already detailed above. The same fact will obviously hold true in the case of any of its substitutes dependent upon acid digestion.

Further, the potassium carbonate solvent is capable of dissolving phosphorus in organic combination in humus, which citric acid solution fails to do. It is well known that phosphorus in organic combination generally remains in a colloidal and highly dispersed state in the soil solution, and, as such, is believed to be more efficacious and easily available to plants than inorganic phosphates in soils (8). Thus it may perhaps be maintained that potassium carbonate solution, which extracts phosphorus in both organic and inorganic combinations in soils, is a very suitable solvent for the estimation of soil phosphates which will be available for plant nutrition. Consequently, the potassium carbonate method is capable of measuring the probable fertility of all types of soil with respect to available phosphoric acid, and, as such, can be recommended as of universal application for this purpose. Besides, citric acid solution extracts, in the majority of cases, especially in calcareous soils, very small amounts of phosphoric acid, giving rise to great manipulative difficulties in their estimation. Thus the potassium carbonate method is a decided improvement on the existing citric acid method.

SUMMARY AND GENERAL CONCLUSIONS

A large number of typical soils of known cropping and manurial history, consisting of acid, laterite, humus, alkali, calcareous, and non-calcareous ones, were collected from different parts of India for a comparison of the new potas-

TABLE 6

Comparison of total and available phosphoric acid by citric acid and potassium carbonate methods in non-calcareous soils of known cultural history

DESCRIPTION OF PLOTS	CROPS GROWN	PER CENT TOTAL P_2O_5 (T)	PER CENT AVAILABLE P_2O_5		$\frac{K}{T}$	$\frac{C}{T}$
			By citric acid (C)	By K_2CO_3 (K)		
Coimbatore Permanent Experimental Plots, Madras						
No manure	Sorghum, ragi (*Eleucine coracana*), and wheat	0.0428	0.0118	0.0010	0.022	0.276
P	Sorghum, ragi (*Eleucine coracana*), and wheat	0.0790	0.0600	0.0016	0.020	0.759
N	Sorghum, ragi (*Eleucine coracana*), and wheat	0.0516	0.0138	0.0010	0.025	0.267
N + P	Sorghum, ragi (*Eleucine coracana*), and wheat	0.1515	0.0917	0.0026	0.017	0.606
N + K	Sorghum, ragi (*Eleucine coracana*), and wheat	0.0845	0.0455	0.0016	0.019	0.538
N + K + P	Sorghum, ragi (*Eleucine coracana*), and wheat	0.1421	0.0847	0.0026	0.018	0.596
Cattle manure	Sorghum, ragi (*Eleucine coracana*), and wheat	0.0463	0.0139	0.0016	0.034	0.295
Cattle manure, residual, manuring discontinued since 1916.	Sorghum, ragi (*Eleucine coracana*), and wheat	0.0308	0.0131	0.0017	0.055	0.427
Nandyal Agricultural Station, Madras						
No manure	Sorghum and cotton	.....	0.0034	0.0005	.....	.....
Cattle manure	Sorghum and cotton	.....	0.0300	0.0014	.....	.....
No manure	Sorghum and cotton	.....	0.0065	0.0005	.....	.....
Cattle manure	Sorghum and cotton	.....	0.0111	0.0010	.....	.....

Koilpatti Agricultural Station, Madras						
No manure	Cumbu (*Pennisetum typhoideum*)	.....	0.0074	0.0001	.....	.....
Superphosphate	Cumbu (*Pennisetum typhoideum*)	.....	0.0096	0.0010	.....	.....
Superphosphate + cyanamide	Cumbu (*Pennisetum typhoideum*)	.....	0.0097	0.0010	.....	.....
Dharwar Farm, Bombay						
Non-phosphated	Cotton and tobacco	.....	0.0001	0.0011	.....	.....
Phosphated	Cotton and tobacco	.....	0.0006	0.0012	.....	.....
Gokak Canal Farm, Bombay						
Non-phosphated	Sugarcane	.....	0.0258	0.0039	.....	.....
Phosphated	Sugarcane	.....	0.0397	0.0058	.....	.....
Dohad Farm, Panch Mahals, District Guzerat, Bombay						
Plot no. 70, good plot	Maize and gram	.....	0.0120	0.0020	.....	.....
Plot no. 71, supered in 1914	Maize and gram	.....	0.0064	0.0016	.....	.....
Indore soil, Central India						
Light cotton soil, unmanured	Cotton	.....	0.0018	0.0001	.....	.....
Light cotton soil, fertile	Cotton	.....	0.0070	0.0028	.....	.....
Deep cotton soil, unmanured	Cotton	.....	0.0015	0.0009	.....	.....
Deep cotton soil, manured	Cotton	.....	0.0085	0.0026	.....	.....
Etwah Government Farm, U. P.						
No manure	Gram, peas, and wheat	.....	0.0518	0.0083	.....	.....
Castor cake	Gram, peas, and wheat	.....	0.0379	0.0119	.....	.....
Farmyard manure	Gram, peas, and wheat	.....	0.0233	0.0044	.....	.....

TABLE 6—*Concluded*

DESCRIPTION OF PLOTS	CROPS GROWN	PER CENT TOTAL P_2O_5 (T)	PER CENT AVAILABLE P_2O_5		$\frac{K}{T}$	$\frac{C}{T}$
			By citric acid (C)	By K_2CO_3 (K)		
Atara Government Farm, District Banda, U. P.						
Kabar soil, no manure	Wheat and paddy	.....	0.0941	0.0274	.....	.....
Rakar soil, no manure	Gram and sorghum	.....	0.0115	0.0014	.....	.....
Mar soil, no manure	Wheat and gram	.....	0.0164	0.0052	.....	.....
Parwa soil, green manure	Wheat and sorghum	.....	0.0369	0.0147	.....	.....
Lyallpur Agricultural College Farm, Punjab						
Control	Cotton, maize, and senji fodder (*Melilotus parviflora*)	.....	0.0831	0.0033	.....	.....
Bonemeal	Cotton, maize, and senji fodder (*Melilotus parviflora*)	.....	0.0911	0.0038	.....	.....
Gurdaspur Agricultural Station, Punjab						
Field C/6, plot no. 3, control	Wheat	.....	0.0113	0.0041	.....	.....
Field C/6, plot no. 2, super	Wheat	.....	0.0175	0.0062	.....	.....
Field C/6, plot no. 6, complete manure	Wheat	.....	0.0210	0.0066	.....	.....
Field A/10, plot no. 11, control	Striped sugarcane	.....	0.0246	0.0049	.....	.....
Field A/10, plot no. 16, super	Striped sugarcane	.....	0.0311	0.0081	.....	.....
Field A/10, plot no. 10, complete manure	Striped sugarcane	.....	0.0286	0.0069	.....	.....
Field A/1a, plot no. 11, control	Striped sugarcane	.....	0.0150	0.0059	.....	.....
Field A/1a, plot no. 16, super	Striped sugarcane	.....	0.0277	0.0117	.....	.....
Field A/1a, plot no. 10, complete manure	Striped sugarcane	.....	0.0165	0.0075	.....	.....

Cuttack Farm, Orissa						
Plot A, best land	Paddy	.....	0.0051	0.0074	.....	.....
Plot B, medium land	Paddy	.....	0.0036	0.0068	.....	.....
Plot C, poor land	Paddy	.....	0.0009	0.0036	.....	.....
Government Farm, Adhartal, Jubbalpore, C. P.						
Unmanured	.....	.....	0.0013	0.0021	.....	.....
Sann-hemp	.....	.....	0.0010	0.0024	.....	.....
Sann-hemp + superphosphate	.....	.....	0.0012	0.0026	.....	.....
Sabour Government Farm, South Bhagalpur, Bihar						
Poor land, unmanured	Gram and linseed	.....	0.0191	0.0052	.....	.....
Fertile land, unmanured	Gram and linseed	.....	0.2822	0.0172	.....	.....
Ammonium sulphate	Sugarcane	.....	0.1305	0.0171	.....	.....
Kharhara Farm, South Bhagalpur, Bihar						
Unmanured	Paddy	.....	0.0008	0.0019	.....	.....
Ammonium sulphate	Paddy	.....	0.0016	0.0023	.....	.....
Nawada Government Farm, District Gaya, Bihar						
I. Unmanured, poor crop	Gram	.....	0.0004	0.0005	.....	.....
II. Radio-phos (apatite)	Yield better than I	.....	0.0006	0.0008	.....	.....
III. Super	Yield better than I and II	.....	0.0012	0.0012	.....	.....
Residual effect of super	Yield better than I and II	.. ..	0.0011	0.0028	.....	.....

sium carbonate method with Dyer's citric acid method for the estimation of available phosphoric acid. The following conclusions are summarized from the results obtained:

1. The results obtained showed that the potassium carbonate method is equally applicable to all types of soil, whereas the citric acid method breaks down as a discriminating agent for evaluating the available phosphoric acid of alkali and calcareous soils.

2. Besides the obvious advantage of a more general application, the potassium carbonate method possesses several other points in its favour and should therefore replace the existing Dyer's method or any of its substitutes dependent upon acid digestion for estimating the available phosphoric acid of soils.

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NITROGEN FIXATION IN FIELD SOIL UNDER DIFFERENT CONDITIONS OF CROPPING AND SOIL TREATMENT

LEON A. BRADLEY AND JAMES E. FULLER

Massachusetts Agricultural Experiment Station

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The importance of investigating the relationship of bacterial activities in the soil to crop production was emphasized by Brown (2). Perhaps it is equally important to know what influence the crop may have on the bacteria, in order that the complete cycle may be understood. Since it is recognized that certain bacteria, such as nitrifying, nitrogen-fixing, and cellulose-decomposing species, are necessary aids to soil fertility, the complete understanding of such a cycle might make it possible to utilize these microscopic agents more effectively.

The following are a few representative illustrations from the literature dealing with the effects of crops on the microscopic soil flora:

Hiltner (5) stated that growing plant roots stimulate the activities of free-living nitrogen-fixing bacteria. Heinze (4) found that Azotobacter appears to be more widely distributed in cultivated than in virgin soil.

Greaves (3) reported the analyses of hundreds of samples of cultivated and virgin soils in Utah and concluded that virgin soils have a low nitrogen-fixing power as compared with cultivated soils. Brown (2) investigated the effect of crop rotation over a few seasons. His results indicated that a rotation of corn and clover resulted in more active nitrogen-fixation than did corn alone. A rotation of corn and cowpeas had little effect, whereas the rotation of corn with rye apparently depressed nitrogen fixation.

Starkey (14) investigated some influences of the development of higher plants upon the microörganisms in the soil. His article is cited especially because it contains a considerable bibliography on the subject.

EXPERIMENTAL

The present investigation is closely connected with a field experiment which has been in progress for some years at the Massachusetts Agricultural Experiment Station on the effects of different crop rotations and fertilizer treatments on crop production. Some of the results of these field experiments have been published by Ruprecht (10), by Ruprecht and Morse (11, 12), and by Morse (6, 7, 8, 9).

The field, designated as field A, is divided transversely from east to west into six strips, each 2 rods wide, which are numbered 5 to 10 inclusive. The original field included four additional strips, 1 to 4 inclusive, but the construction of a new building a few years ago made their abandonment necessary. Strip 10 is across the north end of the field. The field is further divided longitudinally from north to south into four strips each 2 rods wide.

The first longitudinal strip (beginning on the west side of the field) and the third are planted certain years with a legume crop such as soybeans or clover, and the second and fourth are planted with a non-legume such as millet. On alternate years, the whole field is planted with a non-legume crop, corn being used frequently. Thus, the west and east halves of the field are exact duplicates so far as crops are concerned.

The longitudinal strips are designated in this report by the letters *LW* (legume west), *NLW* (non-legume west), *LE* (legume east), and *NLE* (non-legume east). The transverse and longitudinal divisions of the field result in 24 plots each 2 rods square, each of which is designated by the letter of the longitudinal and the number of the transverse strips, as 10 NLW and 6 LE.

TABLE 1

The arrangement and fertilizer treatment of field A

North

	LEGUMES WEST	NON-LEGUMES WEST	LEGUMES EAST	NON-LEGUMES EAST
10		Dry ground fish		
9		No nitrogen		
8		Ammonium sulfate		
7		No nitrogen		
6		Ammonium sulfate until 1922 No nitrogen since		
5		Sodium nitrate		

The field has been used for a continuous experiment on the effects on crops of different plant nutrients, including nitrogen, potash, phosphorus, and lime compounds. Strip 5 has been treated annually with sodium nitrate, strip 8 with ammonium sulfate. Strip 10 was formerly treated with dried blood, and more recently with dried ground fish. Strip 6 was formerly treated with ammonium sulfate but has had no nitrogen since 1922. Strips 7 and 9 have not received any kind of nitrogenous fertilizer since the experiment was begun in 1882. The whole field is treated annually with uniform quantities of potash and phosphorus. The lime treatment is shown in table 4. The treatment of the field and its influence on crops will be found in the articles by Morse (6, 7, 8, 9) and by Ruprecht and Morse (11, 12). The present experiment has been carried out along similar lines since 1923.

Table 1 shows the arrangement of the field and the nitrogen treatment. As a result of the fertilizer treatment and the system of cropping, the 24 individual plots fall into the following groups: those planted with legumes and receiving

nitrogen, those planted with legumes but receiving no nitrogen, those planted with non-legumes and receiving nitrogen, and those planted with non-legumes and receiving no nitrogen.

At the end of each season, the crops are weighed and analyzed for nitrogen content; nitrogen determinations are also made on the soil from time to time. The department of chemistry of the Massachusetts Agricultural Experiment Station has had charge of the experiments outlined in the foregoing and some of the analyses have been made by the department of feed control.

The outstanding results of the crop and soil analyses have been as follows: 1. More nitrogen has been taken off the field with the crops than has been added as fertilizer. 2. Plots which have not received nitrogen but have been planted with legumes have produced as good crops as any of the others. 3. When crops of Japanese millet have been grown on the field, and in some seasons together with corn crops, the yield has been uniformly good over the whole field, including plots which have had no nitrogen added and no legume crops grown on them.

It is the purpose of this investigation to determine what effect the crop rotation and fertilizer treatment may have on nitrogen-fixation, and also whether the nitrogen-fixation is sufficiently active to account for the residual nitrogen observed in parts of the field which have had neither legume crops or fertilizer treatment.

The experimental work has been conducted according to the following four plans:

I. A comparative study of the bacterial flora of the plot soils in field A by quantitative plating on different media and the subsequent isolation in pure culture of the predominating types of microorganisms.

II. A study of the ability of the organisms isolated in plan I to fix atmospheric nitrogen.

III. An investigation of the relative nitrogen-fixing ability of the soils of the plots of field A.

IV. Determination of the hydrogen-ion concentration of the soils of the plots of field A, and its possible relation to the nitrogen fixation observed in these plots.

Samples of soil for examination were collected in glass-stoppered bottles and also in 8-inch flower pots lined with a coating of paraffine. The bottled soils were taken to the laboratory for immediate examination. The moisture content of the soil in the pots was determined and they were stored in a greenhouse for use during the winter months. Every second day the pots were weighed and the loss from evaporation was made up by the addition of sterile distilled water. The bottled soil will be referred to, in this report, as series B; that in the pots will be designated as series P.

I. Bacterial flora of plot soils in field A

The first experiment undertaken was a quantitative study of the flora of the different plots of field A. Composite soil samples were taken from plots receiving nitrogen and growing legumes, from plots receiving nitrogen but not

growing legumes, from plots not receiving nitrogen but growing legumes, and from plots not receiving nitrogen and not growing legumes.

The media used for plating were Ashby's agar, meat-extract peptone agar, dextrose peptone agar, and soil-extract agar.

Ten grams of soil were added to 90 cc. of sterile physiological salt solution and shaken in a machine. Preliminary investigation suggested dilutions as follows: 1–100,000, 1–250,000 and 1–500,000. Quadruplicate plates were incubated at 25°C., and for six days where possible. Because of spreaders which obscured colony formation, meat-extract and peptone agar plates were incubated for only three days. The results, shown in table 2, indicate that the variations are inconsistent and, for any given medium, they are within the range of experimental error. They probably may be accounted for by biological variation, and are without significance.

TABLE 2

Numbers of bacteria in various soil combinations on different culture media

Millions of bacteria to a gram of dry soil

MEDIUM	BACTERIA IN SOIL COMBINATIONS*			
	N L	N *N-L*	*N-N* L	*N-N N-L*
Ashby's agar, Series B	4.06	5.44	5.25	4.06
Ashby's agar, Series P	8.75	6.00	9.00	11.50
Meat-extract peptone agar, Series B	8.57	7.07	8.90	10.42
Meat-extract peptone agar, Series P	4.23	7.88	3.10	1.93
Dextrose peptone agar, Series P	2.88	2.75	3.25	3.41
Soil-extract agar, Series P	3.18	1.94	4.00	3.50

* N L indicates nitrogen treatment and legume crop. N *N-L* indicates nitrogen treatment but non-legume crop. *N-N* L indicates no nitrogen but a legume crop. *N-N N-L* indicates no nitrogen and non-legume crop.

II. Nitrogen-fixing ability of the organisms isolated from I

A number of organisms, including several actinomyces, were isolated in pure culture from the plates in experiment I. The nitrogen-fixing ability of these organisms was tested as follows:

A heavy inoculum was placed in flasks containing a layer of sterile Ashby's agar and incubated at 25° to 28°C. for three or four days until growth was well established. Fifty cubic centimeters of sterile Ashby's solution were added aseptically and the cultures incubated at 25° to 28°C. for 14 days. This method was advocated by Ashby (1). At the end of the incubation period, total nitrogen determinations were made by the Kjeldahl method. One organism, designated as 9A, was found to fix nitrogen vigorously, the quantity averaging 10 to 12 mgm. to 100 cc. of solution, and in some cases as much as 16 mgm. to 100 cc.

The morphology of the organism, 9A, is typical for Azotobacter. In Ashby's solution or on Ashby's agar, cultures up to 1 week old show a predominance of

diplococcus forms, somewhat granular when stained with erythrosin or rose bengal. The cells are slightly smaller than those of three stock strains of *Azotobacter chroococcum* carried in the laboratory.

When cultivated in Ashby's solution, 9A forms a slightly wrinkled scum which varies in color from yellow to a light brown. On Ashby's agar slants, the organism produces a profuse, slimy growth. At first there is no pigment, but at the end of a week the growth becomes yellow, and darkens to a light brown in old cultures. The pigment never becomes as dark as that of the three stock cultures mentioned.

Colonies on Ashby's agar plates are large, frequently measuring 10 mm. in diameter. They are raised and slimy with a dense center. The coloration is like that of the slant cultures on Ashby's agar.

The writers feel that the organism undoubtedly belongs to the genus Azotobacter.

None of the other organisms fixed nitrogen in measurable quantity, although some were able to grow on nitrogen-free Ashby's medium.

III. Nitrogen-fixing ability of soil in plots of field A

The next experiment undertaken was the determination of the nitrogen-fixing ability of the soil in the different plots of field A, and the prevalence of organisms capable of fixing marked quantities of nitrogen.

Soil samples were taken from the storage pots and well mixed but not dried, the moisture content being maintained as near as possible to that of the soil when taken from the field. One-gram portions were put into 50 cc. quantities of Ashby's solution and incubated at 28°C. Controls were set up in the same manner and immediately sterilized at 15 pounds for 20 minutes, after which they were handled in exactly the same way as the cultures.

At first, incubation for three weeks was attempted but it was found that during the third week putrefaction was so rapid that the cultures were spoiled for nitrogen-fixation tests. Two weeks' incubation was employed satisfactorily for the remainder of the tests. At the end of one week some of the scum growing on the surface of the cultures was plated in Ashby's agar to determine the possible presence of nitrogen-fixing bacteria, especially of the Azotobacter group.

After two weeks' incubation, total nitrogen determinations were made on the cultures and controls by the Kjeldahl method.

Table 3 shows the results of these tests. One series of 1926 and one of 1928 are included as representative of the determinations. During the series of 1928, pure cultures of 9A were isolated from the plots thus indicated and were tested for nitrogen-fixing power, using Ashby's solution and incubating for three weeks at 28°C; Total nitrogen was determined by the Kjeldahl method as before. In all cases, it was found that the organism was capable of fixing substantial amounts of nitrogen. No other aerobic organism was found which could fix measurable amounts of nitrogen, although some were isolated which

could live on the nitrogen-free Ashby's medium. No isolations of anaerobic organisms were attempted.

TABLE 3

Nitrogen-fixation by soils of plots in field A

Milligrams of nitrogen to a gram of soil

North

	DATE	NITROGEN FIXED				
		Legumes West	Non-legumes West	Legumes East	Non-legumes East	
10	November, 1926	4.9+	2.4	4.7+	4.4+	Dry ground fish
		5.3	1.8	5.1	4.4	
	November, 1928	4.3+	2.3	3.0+	3.5+	
		2.7	1.3	3.8	4.0	
9	November, 1926	4.7+	1.8+	4.7+	5.4+	No nitrogen
		4.8	4.9	4.9	6.8	
	November, 1928	3.7+	2.3	4.6+	4.6+	
		3.0	1.3	3.9	3.7	
8	November, 1926	1.9	2.3	4.2+	3.6+	Ammonium sulfate
		2.1	2.6	5.4	4.7	
	November, 1928	1.5	2.1	2.2	4.5+	
		2.0	1.3	0.0	2.4	
7	November, 1926	2.2	2.1	4.8+	6.3+	No nitrogen
		2.0	2.9	4.6	4.8	
	November, 1928	3.3+	4.2+	4.5+	4.1+	
		4.0	1.9	3.4	3.7	
6	November, 1926	2.0	2.5	3.1	5.8	No nitrogen
		2.3	1.7	2.1	3.2	
	November, 1928	2.2	1.4	3.6	3.1	
		1.9	1.7	1.4	4.1	
5	November, 1926	2.2	2.1	4.2	2.2	Sodium nitrate
		2.3	1.9	1.8	2.9	
	November, 1928	2.0	4.4	2.1	4.4+	
		1.3	1.0	1.8	3.9	

The plus mark indicates the presence of organism 9A.

Table 3 shows the following results:

In general, the soil of the east half of the field has a greater nitrogen-fixing ability than that of the west half. Two apparent influences might account for this condition: the slope is

from west to east which might mean a more favorable moisture condition for Azobacter in the east half; and more lime has been added to the east half over a period of 20 years. The amounts of lime added to the plots are shown in table 4. A study of the pH of the different plots was made in order to investigate the possible relation of the liming to the soil acidity. This study is reported later in the manuscript.

In general, the plots showing the highest nitrogen fixation also yielded cultures of 9A. This correlation was expected. The outstanding exception was in plot 6 NLE which had a high nitrogen fixation but did not show any 9A.

The poorest nitrogen fixation was observed in strips 5, 6, and 8 which had received inorganic nitrogen compounds as fertilizer. Ammonium sulfate might be expected to lower

TABLE 4

Total lime applied to field A since 1898

Pounds an acre

North

	LIME ADDED			
	Legumes West	Non-legumes West	Legumes East	Non-legumes East
10	10,000	10,000	18,000 17,000	18,000 17,000
9	10,000	10,000	18,000 17,000	18,000 17,000
8	10,000	10,000	18,000	18,000
7	8,500 7,000	8,500 7,000	17,000 15,500	17,000 15,500
6	10,000	10,000	17,000	17,000
5	10,000	10,000	18,000 17,000	18,000 17,000

Note: The presence of two quantities in some of the squares indicates that the north and south halves of these squares have had different amounts of lime applied at certain periods.

the pH of the soil, but table 5 shows that the liming of the strips apparently had overcome any such influence. Another possible explanation is that suggested by Winogradsky(15): that other microörganisms present in the soil are stimulated by the presence of readily available organic nitrogen, and, because they grow more rapidly than the Azotobacter, they are able to exhaust the available supply of food, especially energy material. Strip 10 has been treated with complex organic fertilizer (dried blood and later dry ground fish) which probably is made available more slowly for microörganic consumption. Under these conditions, the Azotobacter seems to be able to fulfil its nitrogen-fixing function more successfully.

The presence of legume crops on the plots apparently has not influenced nitrogen fixation or the growth of 9A in the soil, since some of the legume plots showed as much fixation as any of the plots in the field.

IV. The pH of the soils of plots in field A

The soil reaction was tested by the potentiometer method, calomel and gold-leaf electrodes being used. Methods for determining hydrogen-ion concentration of soils, later compiled by Snyder (13), were used as a basis for the tests. Table 5 shows the pH determinations in 1926–27 and 1928–29. It will be observed that the figures for 1926–27 are slightly higher than those for 1928–29. This is probably because the field was last limed in the spring of 1926 and the effects had disappeared slightly in the two years elapsing between the tests.

TABLE 5

The pH of soil in plots of field A

The upper figures in each square indicate the determination made in 1926, and the lower figures, those in 1928.

North

	LEGUMES WEST	NON-LEGUMES WEST	LEGUMES EAST	NON-LEGUMES EAST
	pH	*pH*	*pH*	*pH*
10	6.5	6.7	6.7	6.7
	5.8	5.5	6.1	6.2
9	6.6	6.5	6.6	6.6
	5.8	5.7	5.7	5.8
8	...	6.2	6.5	6.4
	5.8	5.8	5.7	5.7
7	6.5	6.4	6.5	6.5
	6.0	6.2	6.2	6.0
6	6.2	6.2	6.4	6.3
	5.8	5.8	6.0	6.0
5	...	6.1	6.1	6.1
	5.9	5.7	6.0	5.9

The lowest pH, 5.5, was obtained in plot 10 NLW, which gave poor nitrogen-fixation figures and did not yield any 9A. However, the same results were observed in 1926 when the pH was 6.5 in this plot. On the whole, it seems that the pH does not explain the results obtained either in regard to nitrogen fixation or to the presence of 9A. In practically all cases, the pH was within the limit of tolerance for the growth and activity of 9A as determined by experiment in this study. The determinations were made from soil that had been stored in pots. It has been stated (13) that storage has a tendency to increase the acidity of soil; therefore the field conditions of the plots are probably more favorable than those of pots for Azotobacter growth and activity.

SUMMARY

Results suggest that there is sufficient nitrogen fixation in the soil of field A to account for the nitrogen reserve.

The nitrogen fixation observed in the plots is correlated with the presence of an Azotobacter strain designated as 9A.

Nitrogen fixation and the distribution of organism 9A appear to have remained reasonably constant over a 3-year period.

The growth of different crops, including legumes and non-legumes, has not influenced nitrogen fixation or the presence of organism 9A in the field.

Plate counts of the organisms in the soil revealed no significant information.

The soil reaction in the plots of field A does not appear to be a controlling factor in the nitrogen-fixing activity and the distribution of organism 9A; this organism tolerates a lower pH than that commonly accepted as a limiting factor for Azotobacter growth.

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ACTINOMYCETES IN DANISH SOILS

H. L. JENSEN[1]

State Laboratory of Plant Culture, Lyngby, Denmark

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The actinomycetes have long been recognized as a constituent of the normal soil microflora. Beijerinck (1) was one of the first to call attention to the fact that the soil harbors a large number of actinomycetes, to the activities of which he ascribed the formation of humus. Soon afterward, Hiltner and Störmer (6) pointed out that these organisms often comprise 20 to 30 per cent of the total number of colonies appearing on gelatin plates in the making of bacterial counts from normal soils.

The first serious attempt to classify the actinomycetes (apart from the pathogenic forms) was not done until 1914, by Krainsky (8), but since then several contributions to an analysis of the actinomyces flora of the soil have been made by Waksman and Curtis (11, 16) and by Conn (2, 3, 4), besides many works on the general morphology and biology of the actinomycetes [for a review of the literature see Lieske (9), Waksman (11), and Ørskov (10)]. About 40 new species have been described by the aforementioned writers, and a good deal of information concerning the rôle of these organisms in biological soil processes has been gained. A great difficulty has been represented, however, by their extreme variability, which is strongly emphasized by all modern authors, especially Lieske (9), who in his very complete treatise on the morphology and biology of the actinomycetes expresses an entire disbelief in the possibility of classifying these organisms as "species." A somewhat similar, although less extreme, view is expressed by Waksman (11), who points out the possibility of classifying them as "species-groups," that is, groups composed of a number of strains showing agreement in their most characteristic morphological and (especially) biochemical features. The present paper represents an attempt to obtain some information about the actinomyces flora of Danish soils, with special reference to the possibility of distinguishing between species-groups, in the sense of Waksman.[2]

[1] Now bacteriologist to the Linnean Society of New South Wales, Sydney.

[2] The work was originally planned as a more comprehensive study, but had to be left because of the writer's departure for Australia; this paper, together with one previously published (7), represents a summary of the preliminary results. The author's thanks are due to Dr. Selman A. Waksman, New Jersey Agricultural Experiment Station, for supplying a number of authentic cultures for comparison with those isolated from the Danish soils.

COUNTINGS OF ACTINOMYCETES IN VARIOUS SOILS

Counts of bacteria and actinomycetes were carried out on the following agar medium: dextrose, 2 gm.; casein dissolved in 10 cc. 0.1 *N* NaOH, 0.2 gm.; K_2HPO_4, 0.5 gm.; $MgSO_4$, 0.2 gm.; $FeCl_3$, trace; agar, 15 gm.; distilled water, 1,000 cc.; pH 6.5 to 6.6. Dilutions of 1:10,000–100,000 were used according to the character of the soil. Six parallel plates were usually poured, and incubated at 25°C. for 7 days. This medium was found eminently suitable for counting and isolating the actinomycetes, and because of the thin growth, which most actinomycetes produced on it, it is also very convenient when a direct microscopical examination of the living colonies is desired; for the cultural distinction of the various strains it was found less suitable. The results of the countings are found in table 1.

It will be seen that the absolute numbers of actinomycetes, which vary from none to about 13 millions per gram, are not materially different in the different kinds of soils of similar reaction; but the reaction has a marked influence on the numbers. All the strongly acid soils, of pH 4.6 and less, have, with one single exception (forest soil 31), only very small numbers of actinomycetes. At a pH value of about 5 the numbers begin to rise, and the maximal figures occur in the region of pH 6.8 to 8.0. This is in perfect agreement with the results of Gillespie (5), Waksman and Joffe (17), and Waksman (14), who found that the critical degree of acidity for the majority of actinomycetes is pH 4.8 to 5.0, and that their optimal reaction is pH 7 to 8. Waksman (13), moreover, found the percentage of actinomycetes decreasing in strongly acid soils. This is here true only in special cases; all the typical acid peat soils—forest soils 28 and 30, moor soils 38 to 41, heath soils 49 and 50—have remarkably low percentages of actinomycetes, but otherwise there is no correlation whatever between the pH values and the percentages of actinomycetes in the total flora on the plates. Even such extremely acid soils as 1, 2, and 27 have the same percentages as the neutral and alkaline soils. Furthermore, the percentages are mostly of the same order of magnitude as those found by Hiltner and Störmer (6), Waksman and Curtis (16), and Conn (2), although some excessive figures occur —soils 14, 19, 23, and 54—which were only found by Waksman and Curtis in certain subsoils.

The soil reaction thus to a marked extent controls the development of actinomycetes in the soil. This statement seems to be no more applicable to actinomycetes than to the bacteria (or at least to those bacteria which develop on the agar medium), since the relative numbers of actinomycetes were affected only in special cases, viz., in the strongly acid peat soils. It must be left to further investigations to decide what is the cause of the relative absence of actinomycetes in this soil type. It is possible that the aeration, which is usually poor in these soils, might play a rôle; however, the results recorded in table 2, where an acid peat soil was kept in a moist, loose, and well-aerated condition, do favor this hypothesis.

As a further test of the influence of reaction on numbers of actinomycetes, the following experiment was carried out: Three more or less acid soils received additions of $CaCO_3$ (the strongly acid peat soil, 4 per cent; the two

TABLE 1

Numbers of actinomycetes in various soils

ANALYSIS NUMBER	SOIL	ACTINOMYCETES, MILLIONS PER GM. OF SOIL	ACTINOMYCETES, PER CENT OF TOTAL	pH OF SOIL
	Field soils			
1	Humus, permanent grass	0.12	29.3	3.76
2	Clay	0.02	20.1	3.97
3	Clay	0.24	13.9	4.96
4	Sand	1.82	40.8	5.09
5	Sand	2.72	23.7	5.56
6	Loam	2.37	32.0	5.92
7	Humus	8.11	42.1	6.09
8	Loam	2.80	25.9	6.22
9	Loam	3.51	18.3	6.70
10	Sand	2.22	14.9	6.78
11	Loam	5.83	24.3	6.85
12	Loam	3.79	22.7	6.86
13	Sand	3.53	15.7	6.91
14	Sand	8.32	66.0	6.93
15	Sand	2.87	16.3	6.98
16	Sand	2.56	16.3	7.03
17	Loam	3.68	20.9	7.15
18	Sand	2.51	15.9	7.16
19	Humus	4.03	51.7	7.20
20	Loam	1.12	10.3	7.23
21	Loam	3.47	16.9	7.26
22	Loam	2.76	17.1	7.32
23	Sand	12.82	73.3	7.40
24	Loam	4.12	26.0	7.50
25	Loam	3.90	24.7	7.52
26	Loam	3.24	29.2	8.35
	Forest soils			
27	Mold	0.04	23.8	3.34
28	Peat	0.01	1.9	3.86
29	Mold	0.07	9.8	4.12
30	Peat	0	0	4.32
31	Mold	1.70	43.7	4.45
32	Sand	1.07	23.5	4.97
33	Clay	1.76	24.8	5.54
	Forest soils—Concluded			
34	Sand	1.68	38.2	5.65
35	Mold	0.54	8.4	6.06
36	Mold	0.51	8.6	6.32
37	Loam	6.37	23.8	7.75
	Moor soils			
38	Sphagnum peat	0.002	0.2	3.62
39	Mud	0.003	0.3	4.17
40	Sphagnum peat	0.01	2.5	4.24
41	Low-moor peat	0.07	4.6	4.26
42	Sphagnum peat, cultivated	0.21	19.9	4.58
43	Sphagnum peat, cultivated	0.18	4.3	5.14
44	Low-moor peat	1.19	33.5	5.20
45	Low-moor peat, cultivated	0.81	24.0	5.64
46	Meadow	6.76	39.1	6.90
47	Fen	4.00	15.4	7.08
48	Low-moor peat	9.67	24.6	7.61
	Heath soils			
49	Dry peat	0	0	3.85
50	Dry peat	0.01	3.0	4.10
	Marsh soils			
51	Clay	2.81	28.2	5.12
52	Sandy loam	2.16	25.0	6.12
53	Clay	1.14	24.2	6.82
	Various uncultivated soils			
54	Sand	12.76	58.2	7.55
55	Sand	12.76	24.4	7.95
56	Loam	3.92	6.8	8.08

mineral soils, each 1 per cent), and soil portions with and without lime were kept at 25°C. for about 3 months, during which the moisture content was kept as close to the optimum as possible, and the numbers of actinomycetes were determined several times. The results are given in table 2. They agree perfectly with the expectation: the strongly acid peat soil with the addition of lime shows an enormous increase in actinomycetes: the acid sand soil, poor in organic matter, shows a rather small, although definite increase: and finally the faintly acid loam shows a hardly significant increase. The relative numbers of actinomycetes in the two last soils are distinctly lowered by the lime, because of the very marked stimulation of the bacteria. The enormous development of actinomycetes in acid peat soil with lime speaks strongly in

TABLE 2

Influence of $CaCO_3$ on numbers of actinomycetes in acid soils

SOIL	PERIOD OF INCUBATION	WITHOUT $CaCO_3$			WITH $CaCO_3$		
		Number of actinomycetes*	Actinomycetes per cent†	pH	Number of actinomycetes*	Actinomycetes per cent†	pH
	days						
Heath soil 49	Start	No actinomycetes		3.85	(0)	—	—
	15	on casein agar.		—	18.3	22.0	7.52
	45	*Act. acidophilus*		3.67	84.9	21.1	7.47
	75	(7) present		3.90	122.9	29.6	7.59
Field soil 4	Start	1.8	40.8	5.09	1.8	40.8	—
	15	3.1	61.0	4.71	8.0	35.0	7.57
	90	2.7	57.6	4.42	10.2	23.4	7.50
Field soil 6	Start	2.4	32.0	5.92	2.4	32.0	—
	15	2.7	24.0	6.04	3.0	15.5	7.72
	90	2.9	36.2	5.78	3.5	20.4	7.62

* Millions per gm. of soil.

† Percentage of numbers of bacteria + actinomycetes on plates.

favor of the theory of Waksman (15), that several actinomycetes, if suitable reaction and good aeration are provided, are capable of decomposing soil "humus;" on the other hand we must admit that according to this theory one might expect to find especially large numbers of actinomycetes in neutral and alkaline humus soils. This is, however, not the case (table 1).

CULTURAL STUDIES OF ISOLATED ORGANISMS

About 90 strains of actinomycetes were isolated from the aforementioned soils, and attempts were made to separate them into species-groups and to identify them with previously described forms. The following characters were studied:

I. Presence or absence of spirals in aerial mycelium on dextrose-casein-agar.
II. Cultural characters on the following media:[3]
1. *Czapek's agar.* Saccharose, 30 gm.; $NaNO_3$, 2 gm.; K_2HPO_4, 1 gm.; $MgSO_4$, 0.5 gm.; KCl, 0.5 gm.; $FeSO_4$, trace; agar, 15 gm.; distilled water, 1,000 cc.; pH 7.
2. *Glycerin-agar.* Glycerin 10 gm.; asparagin, 1 gm.; K_2HPO_4, 0.5 gm.; agar, 15 gm.; pH 7.
3. *Starch-casein-agar.* Soluble starch, 10 gm.; casein dissolved in 1 *N* NaOH, 1 gm.; K_2HPO_4, 0.5 gm.; $MgSO_4$, 0.5 gm.; agar, 15 gm.; distilled water, 1,000 cc.; pH 7. This medium was found especially favorable in many cases, allowing a very abundant and yet characteristic growth with strong formation of a characteristically colored aerial mycelium of nearly all strains.
4. *Nutrient agar* with addition of 2 per cent glycerin and 0.1 per cent K_2HPO_4. This medium gave an excellent growth and in many cases a characteristically colored vegetative mycelium and soluble pigments, but often no aerial mycelium.
5. *Gelatin.* 15 per cent gold label gelatin in distilled water; reaction adjusted to pH 7.
6. *Potato plugs.*
7. LOEFFLER'S *Blood Serum.*

All cultures were made as slope cultures in ordinary test tubes and incubated at 25°C. Observations were made for a period of about 6 weeks.

Among the strains of actinomycetes tested in this way the following species-groups could be recognized:

A. No brown pigment in protein-media.
1. Red or blue pigments in synthetic media; marked reduction of nitrates:
Actinomyces violaceus-ruber.
2. No red or blue pigments.
a. Typical golden pigment in all synthetic media:
Actinomyces fulvissimus.
b. Pigment not typical; abundant aerial mycelium.
I. Aerial mycelium on synthetic media dark slate-gray; lemon- or sulfur-yellow pigments sometimes formed.
α. Vegetative mycelium on Czapek's agar light-colored:
Actinomyces cellulosae.
β. Vegetative mycelium on Czapek's agar turning dark:
Actinomyces olivaceus.
II. Aerial mycelium greenish or yellowish gray; very rapid liquefaction of gelatin and blood-serum.
α. Aerial mycelium greenish:
Actinomyces griseus.
β. Aerial mycelium yellowish:
Actinomyces griseoflavus.
B. Typical brown pigment in protein-media ("chromogenus" species).
1. Deep brown growth and pigment in all synthetic media:
Actinomyces pheochromogenus.

[3] A much larger variety of media were tested, but finally those here mentioned were selected as giving the most satisfactory results.

2. Pigment in synthetic media of other color or absent.
   a. Aerial mycelium absent or in traces; typical red vegetative mycelium: *Actinomyces bobili.*
   b. Aerial mycelium more or less abundant.
      I. Typical red pigment in Czapek's agar: *Actinomyces erythrochromogenus.*
      II. Pigment not red.
         α. Aerial mycelium rose to cinnamon-brown: *Actinomyces roseus.*
         β. Aerial mycelium abundant, characteristic lead-grey; light brown pigment in synthetic media: *Actinomyces diastatochromogenus.*

A brief description of these groups is given in the following:

Actinomyces griseus Krainsky, emend. Waksman and Curtis. Six strains + one authentic examined. *Spiral formation:* none. Growth on *Czapek's agar:* Fair; vegetative mycelium scant, thin, first colorless, later yellowish gray; aerial mycelium abundant, thick, dusty layer of a characteristic greenish gray color, sometimes with a yellowish tinge; a faint yellowish gray soluble pigment is sometimes formed. *Glycerin-agar:* Scant growth; vegetative mycelium thin, colorless; aerial mycelium thin, first white, later yellowish green. *Starch-casein-agar:* Abundant and characteristic growth; vegetative mycelium spreading, cream-colored to greenish gray; aerial mycelium formed early and abundantly, thick dusty layer of a strong greenish gray color; olive-green soluble pigment sometimes formed. *Nutrient agar:* Abundant growth; vegetative mycelium cream-colored; aerial mycelium formed after 15 to 20 days, first white, later greenish gray to yellowish green; faint yellowish brown, soluble pigment in old cultures. *Potato:* Strong and characteristic growth; vegetative mycelium abundant, folded, first cream-colored, later yellowish or grayish green; aerial mycelium abundant, dusty, grayish green; plug colored gray. *Gelatin:* vegetative mycelium cream-colored, later greenish gray; aerial mycelium scant, white, later yellowish gray; slant of gelatin completely liquefied in 4 to 6 days; smell of ammonia; no pigment. *Loeffler's blood serum:* uncharacteristic growth; no aerial mycelium or pigment; serum almost completely liquefied in 15 days. Most cultures possess a very strong "soil" odor, some strains a remarkably pungent smell of musty straw. *Occurrence:* common; although not in large numbers.

Actinomyces griseoflavus Krainsky. Two strains examined. *Spiral formation:* none. *Czapek's agar:* good growth; vegetative mycelium flat, spreading, first colorless, later yellowish gray; aerial mycelium abundant, thick, dusty, first white, later characteristic yellowish gray, often forming a mosaic of white and yellowish gray patches; no pigment. *Glycerin-agar:* fair growth; vegetative mycelium flat, first colorless, later pale yellow to yellowish gray; aerial mycelium smooth, first white, later grayish yellow, with net or ring formation; no pigment. *Starch-casein-agar:* very good growth; vegetative mycelium flat, spreading, first colorless, later cream-colored; aerial mycelium abundant, thick, white to grayish yellow with distinct net formation; no pigment. *Nutrient agar:* abundant, but uncharacteristic growth; vegetative mycelium raised, folded, cream-colored to light yellowish brown; aerial mycelium in patches, white to yellowish gray; pigment absent or faint yellowish brown. *Potato:* enormously strong growth, plug after 8 to 10 days completely covered by tufts of mycelium 4 to 6 mm. high; vegetative mycelium raised, folded, cream-colored; aerial mycelium abundant, dusty, white to yellowish gray, sometimes with a pink tinge. *Gelatin:* vegetative mycelium smooth, white to cream-colored; aerial mycelium well developed, white to light yellowish gray; gelatin completely liquefied in 6 to 7 days; no pigment. *Loeffler's blood serum:* uncharacteristic growth; serum largely liquefied in 15 days. *Occurrence:* not common. This group resembles the preceding one very much, but can be distinguished from it by the characteristic grayish yellow of its aerial mycelium which never assumes the distinct green shade of the *griseus* group.

Actinomyces cellulosae Krainsky. Five strains examined. *Spiral formation:* none. *Czapek's agar:* fair and characteristic growth; vegetative mycelium thin, spreading deep into medium, of a characteristic transparent appearance, first colorless, in old cultures sometimes lemon-yellow; aerial mycelium abundant, thick, crusty, often with zone-formation, first light gray, later deep slate gray; pigment mostly absent, sometimes bright lemon-yellow. *Glycerin-agar:* fair, but less characteristic growth; vegetative mycelium narrow, first white, later light gray to yellowish gray or almost black; aerial mycelium abundant, first white, later dark slate-grey or grayish brown; pigment absent or faint lemon-yellow to yellowish brown. *Starch-casein-agar:* good growth; vegetative mycelium smooth, first colorless or cream-colored, later yellowish brown; aerial mycelium variable, often abundant, slate gray; pigment absent or light yellowish brown. *Nutrient agar:* abundant and characteristic growth; vegetative mycelium spreading, much folded, first cream-colored, later sulfur-yellow; aerial mycelium formed late, but becomes abundant, first thin and white, later of the characteristic slate-gray color; no pigment. *Potato:* abundant and characteristic growth; vegetative mycelium raised, folded, cream-colored, later often sulfur-yellow; aerial mycelium abundant, first white, later dark slate-gray, often with yellow edges. *Gelatin:* vegetative mycelium thin, yellowish gray to grayish black; aerial mycelium in patches, grayish white to gray-brown; gelatin completely liquefied in 5 to 8 days; no pigment. *Loeffler's blood serum:* vegetative mycelium cream-colored; aerial mycelium white to light gray; no pigment; serum completely liquefied in 20 days. *Occurrence:* very common.

Actinomyces olivaceus Waksman. Seven strains + one authentic examined. *Spiral formation:* mostly none, sometimes a few imperfect, open spirals. *Czapek's agar:* fair growth; vegetative mycelium thin, spreading into medium, first colorless, later yellowish gray, dark gray, or almost black; aerial mycelium abundant, smooth, dusty, often forming a mosaic of white and yellowish gray patches; pigment absent or light lemon-yellow. *Glycerin agar:* fair growth; vegetative mycelium narrow, growing into medium, first light gray, later black, often with yellow tinge, aerial mycelium abundant, smooth, first white, later dark gray; pigment absent or light yellow. *Starch-casein-agar:* good growth; vegetative mycelium flat, smooth, dark gray; aerial mycelium abundant, smooth, slate-gray, often with mosaic-formation; pigment absent or faint yellow. *Nutrient agar:* good growth; vegetative mycelium raised, spreading, cream-colored; aerial mycelium thin, in patches, white to yellowish gray; faint yellowish pigment. *Potato:* abundant growth; vegetative mycelium raised, folded, cream-colored; aerial mycelium abundant, dusty, grayish white to slate-gray or dark yellowish gray with yellow edges (much like *Act. cellulosae*). *Gelatin:* vegetative mycelium thin, cream-colored to yellowish gray; aerial mycelium thin, gray and white patches; gelatin completely liquefied in 6 to 8 days; no pigment. *Occurrence:* very common. Closely related to the previous group, from which those strains which do not produce an intensely colored growth on Czapek's agar are difficult to distinguish; most characteristic is perhaps the mosaic formation in the aerial mycelium.

Actinomyces violaceus-ruber Waksman and Curtis. Three strains + one authentic examined. *Spiral formation:* numerous spirals. *Czapek's agar:* good growth; vegetative mycelium thin, spreading, first colorless, then red-violet, in old cultures deep blue-violet; aerial mycelium abundant, smooth, light gray; pigment first light pink-violet, later sky-blue. *Glycerin-agar:* good growth; vegetative mycelium thin, first white, with red and violet spots, later deep violet-blue. *Starch-casein-agar:* abundant growth; vegetative mycelium flat, spreading, first pink, later turning deep blue; aerial mycelium in patches, white to grayish blue; pigment dark blue. *Nutrient agar:* abundant growth; vegetative mycelium raised, folded, first cream-colored, later turning deep blue-green; aerial mycelium thin, smooth, white to ash-gray; olive-green pigment in old cultures. *Potato:* good growth; vegetative mycelium spreading, folded, blue; aerial mycelium in patches, gray, turning dark violet; dark blue pigment. *Gelatin:* vegetative mycelium cream-colored with red spots; aerial mycelium scant, white patches; slow liquefaction (complete after 25 days); faint red-brown pigment in old cultures. *Loeffler's blood serum:* vegetative mycelium flat, spreading, violet-blue;

no aerial mycelium; no pigment; trace of liquefaction after 25 days. *Occurrence:* fairly common.

Actinomyces fulvissimus n. sp. Six strains examined. *Microscopical appearance:* vegetative mycelium without any special characteristics; aerial mycelium of short, straight, often trifurcated hyphae, 1.0 to 1.2 μ broad; no spiral formation; branches of hyphae break up into oidia, 1.0 to 1.2 $\times$ 1.2 to 1.5 μ. *Czapek's agar:* good growth (one strain very scant), vegetative mycelium flat, narrow, first light golden, later deep orange to red-brown; aerial mycelium scant, sometimes almost absent, first white, later light grayish brown; pigment very characteristic, bright golden to orange. *Glycerin-agar:* good growth; vegetative mycelium narrow, raised, smooth, golden to dark bronze; aerial mycelium scant, in patches, white to light cinnamon-brown; pigment intensely golden to orange. *Starch-casein-agar:* good growth; vegetative mycelium spreading, folded, yellowish brown; aerial mycelium abundant, smooth, lead-gray; pigment dull yellow to orange. *Nutrient agar:* good growth; vegetative mycelium raised, finely wrinkled, deep red-brown; no aerial mycelium; brownish yellow pigment. *Potato:* good growth; vegetative mycelium raised, much wrinkled, rust-brown; aerial mycelium absent or traces of white; pigment gray to faint lemon-yellow. *Gelatin:* vegetative mycelium narrow, smooth, yellowish brown to red-brown; no aerial mycelium; no pigment; gelatin completely liquefied in 10 to 12 days. *Loeffler's blood serum:* vegetative mycelium red-brown; no aerial mycelium; yellowish pigment; no liquefaction. The characteristic golden pigment is formed in nearly all media in which the organism grows, but becomes most typical and attains its greatest brightness in synthetic agar media; it has indicator properties, turning red in strongly acid solutions. *Occurrence:* extremely common in Danish soils; easily recognized on agar plates by its bronze-colored colonies, surrounded by haloes of bright yellow pigment.

Actinomyces roseus Krainsky, emend. Waksman and Curtis. Six strains + one authentic examined. *Spiral formation:* present, sometimes abundant. *Czapek's agar:* scant to strong growth; vegetative mycelium thin, white, sometimes (e.g. authentic culture from Waksman) with age becoming greenish black with metallic lustre; aerial mycelium thin, sometimes in patches, light rose; no pigment. *Glycerin-agar:* fair growth; vegetative mycelium flat, spreading into medium, white to pale pink, in one strain grayish green; aerial mycelium smooth, rose to cinnamon-brown; no pigment. *Starch-casein-agar:* good growth; vegetative mycelium spreading into medium, yellowish brown to greenish gray; aerial mycelium abundant, thick, smooth layer of a characteristic rose color; no pigment. *Nutrient agar:* abundant growth; vegetative mycelium superficially spreading, cream-colored to red-brown, in two strains of a characteristic green color; aerial mycelium scant, white, formed late; brown pigment. *Potato:* good to abundant growth; vegetative mycelium spreading, flat or folded, yellowish gray to greenish black in two strains; aerial mycelium scant to abundant, first white, later rose-colored; brownish gray pigment. *Gelatin:* vegetative mycelium narrow, folded, yellowish brown to gray-green; aerial mycelium scant, white to light rose; brown pigment; slow liquefaction. *Occurrence:* fairly common, though not in large numbers.

Actinomyces bobili Waksman and Curtis. Six strains + one authentic examined. *Spiral formation:* none. *Czapek's agar:* good growth; vegetative mycelium raised, spreading, somewhat folded, pale rose to coral-red; with colorless edges; no aerial mycelium; no pigment. *Glycerin agar:* good growth, in appearance like the previous, blood-red; no aerial mycelium or only traces of white; mostly no pigment, sometimes pale rose. *Starch-casein-agar:* good, but less characteristic growth; vegetative mycelium superficially spreading, grayish rose, sometimes with small tufts of grayish white aerial mycelium; grayish orange pigment. *Nutrient agar:* abundant and characteristic growth; vegetative mycelium superficially spreading, coarsely folded (lichenoid), characteristically coral-red; no aerial mycelium; deep brown pigment; when glycerin is omitted from the medium, a comparatively scant and quite uncharacteristic yellowish brown growth is obtained. *Potato:* fair growth; vegetative mycelium raised, wrinkled, grayish brown to coral-red; aerial mycelium absent or traces of white; pigment grayish to black. *Gelatin:* vegetative mycelium orange, sometimes with patches of

white aerial mycelium; brown pigment; fairly rapid liquefaction, almost complete after 15 days. *Occurrence:* common soil organism: easily recognized on plates of casein-agar by its colonies which resemble drops of sealing wax.

Actinomyces diastatochromogenus Krainsky. Six strains examined. *Spiral formation:* abundant. *Czapek's agar:* good growth; vegetative mycelium superficially spreading, first colorless, later cream-colored to yellowish brown; aerial mycelium abundant, formed late, first white, later ash-gray; yellowish to light brown pigment. *Glycerin agar:* fair growth; vegetative mycelium spreading into medium, colorless to cream-colored; aerial mycelium less abundant than on previous medium, white to light gray; no pigment. *Starch-casein-agar:* abundant and characteristic growth; vegetative mycelium superficially spreading, yellowish gray; aerial mycelium abundant, smooth, first white, later deep lead-gray, with characteristic net formation; grayish yellow to yellowish brown pigment. *Nutrient agar:* abundant growth; vegetative mycelium spreading, yellowish to grayish brown; aerial mycelium absent or scant, white; light coffee-brown pigment. *Potato:* fair growth; vegetative mycelium spreading, finely wrinkled, first yellowish gray, later grayish black, with or without traces of white aerial mycelium; black pigment. *Gelatin:* vegetative mycelium cream-colored to yellowish brown; aerial mycelium scant, white; brown pigment; gelatin fairly rapidly liquefied. *Loeffler's blood serum:* scant and uncharacteristic growth; dark yellowish brown pigment; no liquefaction. *Occurrence:* very common.

Actinomyces erythrochromogenus Krainsky. Two strains examined. *Spiral formation:* abundant. *Czapek's agar:* fair growth; vegetative mycelium thin, first cream-colored, later turning dark violet to purple; aerial mycelium smooth, light gray; red-violet pigment. *Glycerin-agar:* fair growth; vegetative mycelium narrow, growing into medium, first light red-violet, later turning dark red; aerial mycelium thin, light gray to red-gray; yellowish red pigment. *Starch-casein-agar:* good growth; vegetative mycelium flat, spreading, dark violet; aerial mycelium smooth, light gray; purplish gray pigment. *Nutrient agar:* good growth; vegetative mycelium raised, wrinkled, yellowish gray to light brown; aerial mycelium smooth, light gray; deep brown pigment. *Potato:* good growth; vegetative mycelium raised, wrinkled, first yellowish gray, later almost black; deep black pigment. *Gelatin:* vegetative mycelium finely wrinkled, yellowish to light purple, with thin patches of aerial mycelium; brown pigment; very slow liquefaction. *Occurrence:* not common.

Actinomyces pheochromogenus Conn. One strain examined. *Spiral formation:* present. *Czapek's agar:* good growth; vegetative mycelium growing deep into medium, first red-brown, later nearly black; aerial mycelium well developed, smooth, first pure white, later with brownish tinge; dark brown pigment. *Glycerin agar:* good growth; vegetative mycelium raised, folded, brownish black; no aerial mycelium; brown pigment. *Starch-casein-agar:* like on previous medium, but with thin white aerial mycelium. *Nutrient agar:* good growth; vegetative mycelium raised, smooth, red-brown, later turning almost black; no aerial mycelium; brown pigment. *Potato:* fair growth; vegetative mycelium raised, wrinkled, black; no aerial mycelium; black pigment. *Loeffler's blood serum:* scant growth; vegetative mycelium brownish gray; no aerial mycelium; brown pigment; no liquefaction. *Occurrence:* not common.

Besides these, some strains were found, which probably belonged to or were closely related to the following groups: *Act. aureus* Waksman and Curtis, *Act. Halstedii* Waksman and Curtis, *Act. olivochromogenus* Waksman, and *Act. viridochromogenus* Krainsky. The rest of the cultures—the majority—could not be identified, although several of them could be classified in more or less defined species-groups. The time available for the experiments did not, however, allow a thorough study of these groups, of which only one, viz., the *Act. fulvissimus* already described, was considered sufficiently well defined to be described and named as a new species-group.

PHYSIOLOGICAL STUDIES OF ISOLATED ORGANISMS

Attempts were made to distinguish, by means of the following physiological tests, between the various species-groups recognized through cultural tests, in order to see how far such physiological tests would be of value for the characterization of the non-identified groups: utilization of carbohydrates; acid formation from carbohydrates; reduction of nitrate; diastatic activity; proteolytic activity; resistance to hydrogen-ion concentration.

The utilizaton of carbohydrates was studied by growing the organisms for 10 days[4] at 25°C. on the following solution: carbohydrate, 10 gm. (cellulose supplied as a strip of pure Swedish filter paper); $NaNO_3$, 2 gm.; K_2HPO_4, 1 gm.; $MgSO_4$, 0.5 gm.; distilled water, 1,000 cc. At the end of the experiment, tests were made for NO_2 formation by means of Gries' reagent, acid formation by addition of bromo-thymol-blue (in some cases also by electrometric determination of the reaction), invertase production in saccharose solution by Fehling's reagent, and diastatic activity in starch solution by means of iodine solution.

The utilization of various carbon compounds is shown in table 3. It is seen at once that this gives hardly any differentiation among the groups. Dextrose, galactose, maltose, and starch are the best sources of carbon for nearly all groups, and the only indication of an intergroup distinction is, that the *olivaceus* group hardly utilizes maltose at all and grows poorly on dextrose, and that the *violaceus-ruber* and *diastatochromogenus* groups are the only ones which grow well on lactose and rhamnose. The value of these features, however, seems nullified by the circumstance that the intragroup variation in several cases is equally large, ranging from 0 to 5 in the *griseus* group on arabinose, 2 to 4 in the *olivaceus* group on glycerin and galactose and the *violaceus-ruber* group on dextrose. The table further shows that several strains, especially of the *violaceus-ruber* group, are able to utilize cellulose, although none of the strains here dealt with produced any very considerable growth, even after long incubation. A few non-identified strains, isolated from soil with addition of cellulose, made a good growth and caused a marked disintegration of the filter paper within a few weeks.

The results of the tests for nitrites and acids are given in table 4. The *violaceus-ruber* group shows decidedly the strongest nitrite-formation [as has also been recorded by Waksman (11)]; and in the *griseus*, *griseoflavus*, *cellulosae*, and *olivaceus* groups this property is almost lacking; and, finally, in the rest of the strains it is quite variable and does not characterize any particular group.

The tests for acid formation gave an interesting result. In most papers on actinomycetes (for example, Waksman (12, 17) it is stated that these organisms do not form organic acids from carbohydrates; this was also the case here

[4] Except the cellulose-cultures, which were incubated for 4 months.

TABLE 3

*Utilization of various carbon-compounds by actinomycetes**

SPECIES-GROUP	STRAIN	GLYCERIN	ERYTHRIT	MANNITE	ARABINOSE	XYLOSE	RHAMNOSE	DEXTROSE	LEVULOSE	GALACTOSE	SACCHAROSE	MALTOSE	LAKTOSE	RAFFINOSE	INULIN	STARCH	CELLULOSE
Actinomyces griseus	A. L. VIII.	2	1	4	5	2	0	3	2	3	1	3	0	0	1	4	0
	H. VI.	3	0	3	0	2	1	3	1	3	1	4	1	1	2	5	0
	M. III.	–	1	3	0	0	1	3	1	3	2	2	0	1	2	4	0
	V. III.	3	1	2	0	2	1	3	2	3	1	3	0	0	2	5	0
	W. aut.	2	–	5	2	–	1	4	–	5	5	4	1	1	2	5	–
Actinomyces griseoflavus	A. XVIII.	5	1	5	2	2	1	5	4	5	2	4	2	0	1	5	0
	Hed. IV.	4	1	2	2	2	1	5	4	5	2	5	2	0	2	5	1
Actinomyces cellulosae	Sp. I.	2	1	1	1	2	1	4	4	4	1	4	0	0	1	5	1
	A. V.	5	1	2	3	2	2	3	2	4	2	5	0	1	1	5	2
	A. X.	4	1	2	–	2	0	4	3	3	1	4	0	1	1	4	2
	A. L. III.	3	1	1	1	2	0	3	3	4	1	3	0	0	2	5	1
Actinomyces olivaceus	Sp. III.	3	0	1	0	1	1		1	3	0	1	0	0	–	4	1
	B. XV.	4	0	1	4	2	1	2	1	2	1	0	0	0	1	2	2
	L.	4	1	2	3	0	0	2	0	3	1	1	0	1	2	4	3
	L. M. II.	2	0	–	0	2	0	–	–	4	1	0	0	0	2	5	1
	W. aut.	2	0	2	1	2	0	1	–	4	1	0	0	0	–	5	–
Actinomyces violaceus-ruber	L. II.	3	1	3	3	2	2	4	1	3	1	4	4	2	1	5	3
	L. III.	2	–	3	3	3	3	2	1	3	1	4	3	2	2	4	3
	V. G. III.	2	1	2	2	2	3	3	0	4	2	4	2	3	2	4	1
	W. aut.	1	–	2	2	–	–	2	–	1	1	–	3	1	–	2	–
Actinomyces fulvissimus	H. I.	3	1	1	3	4	0	4	3	3	2	4	0	4	2	3	0
	T. XIV.	2	1	1	3	2	3	4	2	4	3	3	0	3	3	5	0
	H. X.	3	1	1	2	2	0	4	2	1	2	–	3	2	2	4	0
Actinomyces roseus	B. V.	5	0	1	0	0	0	3	1	2	3	3	2	3	2	5	0
	R. IV.	5	1	0	1	0	0	4	1	3	2	4	3	1	1	3	0
	U. II.	–	0	–	–	–	0	–	–	–	4	–	–	3	–	–	1
	W. aut.	1	0	0	3	1	0	3	–	1	1	–	–	0	–	4	–
Actinomyces bobili	U. G.	2	1	3	1	2	2	2	2	3	3	2	2	3	3	2	2
	W. aut.	–	0	1	0	2	1	3	–	3	2	3	3	4	–	3	2
Actinomyces diastatochromogenus	B. IV.	4	0	5	3	2	4	3	1	4	–	4	4	0	5	4	2
	A. XVI.	4	1	4	1	1	3	2	0	–	4	3	3	2	5	4	–
Actinomyces pheochromogenus	H. b.	2	0	1	0	2	1	1	0	1	1	1	1	3	1	1	0

* The amount of growth is here and in the subsequent tables indicated by the following characters: 0 = no growth. 1 = trace of growth. 2 = scant. 3 = fair. 4 = good. 5 = excellent.

TABLE 4

Formation of organic acids from carbohydrates and of NO_2 from NO_3 by actinomycetes in synthetic solution

ORGANISM	ACID FORMED FROM CARBOHYDRATES	NO_2 FORMATION FROM $NaNO_3$	REACTION DETERMINATION IN CASE OF POSITIVE ACID FORMATION		
			Carbohydrate	pH	pH sterile solution
Actinomyces griseus, 5 strains	None	None or trace	–	–	–
Actinomyces griseoflavus, 2 strains	None	None or trace	–	–	–
Actinomyces cellulosae, 4 strains	None	None or trace	–	–	–
Actinomyces olivaceus, 5 strains	None	None	–	–	–
Actinomyces:					
HI, HX, T XIV	None	None or trace	–	–	–
RV	Saccharose, Mannite	None to faint	Saccharose	4.5	7.2
Act. violaceus-ruber:					
L. II	Glycerin, Arabinose, Levulose	Very strong	Glycerin	5.9	7.2
L. III	Glycerin, Rhamnose, Levulose	Very strong	Glycerin	5.8	7.2
			Levulose	6.2	6.6
V. G. III	None	Very strong	–	–	–
Act. roseus:					
B. V	Saccharose, Galactose	None to faint	Saccharose	5.5	7.2
V. II	Saccharose	None to faint	Saccharose	5.5	7.2
Act. bobili:					
V. XII	None	None to faint	–	–	–
V. G	Saccharose	None to distinct	Saccharose	5.3	7.2
Act. Halstedii:					
B. IX	Saccharose	Mostly none, sometimes faint	Saccharose	6.1	7.2
B. XIX	Mannite, Saccharose		Saccharose	6.0	7.2
Act. aureus L. s. (?)	None	None	–	–	–
Act. erythrochromogenus T. IV	Dextrose, Saccharose	None, sometimes faint	Saccharose	5.6	7.2
Act. diastatochromogenus A. XVI	Lactose, Dextrose, Maltose	Strong	Dextrose	6.0	7.2

with most of the cultures, but many of the strains (several of the non-identified besides those here mentioned) show an undoubted formation of acids from various carbohydrates; this acid formation varies both with the type of the organism and the nature of the carbon compound: no strain of the *griseus*, *griseoflavus*, *cellulosae*, or *olivaceus* groups showed any acid formation, and in no case was acid formation observed in solutions of erythrit, inulin, and starch. The formation of acid was constantly accompanied by a more or less vigorus nitrite formation, whereas the reverse did not hold. It is noteworthy that in no case was any turbidity of the cultures, which might suggest bacterial infection, observed.

The results of tests for diastase and invertase production are given in table 5 and show that the *griseus*, *griseoflavus*, *cellulosae* and *olivaceus* groups together with the *fulvissimus* group are the most powerful starch decomposers, although

TABLE 5

Diastase and invertase production in starch and saccharose solution

ORGANISMS	STARCH REACTION	REDUCTION OF FEHLING'S SOLUTION
Act. griseus, 5 strains	None	None
Act. griseoflavus, 2 strains	None	None
Act. cellulosae, 5 strains	None	None
Act. fulvissimus, 3 strains	None	Faint
Act. olivaceus, 5 strains	None	None
Act. violaceus-ruber, 3 strains	Trace or faint	None
Act. diastatochromogenus, 3 strains	None or trace	Strong
Act. bobili, 2 strains	Good	Strong
Act. Halstedii (?), 2 strains	None or trace	–
Act. aureus, 2 strains	Strong	None
Act. pheochromogenus	Strong	None
Act. erythrochromogenus	Trace	–

the *diastatochromogenus*, *Halstedii* (?), and *erythrochromogenus* groups follow them closely, and the rest of the "chromogenus" actinomycetes (*bobili*, *aureus* (?), and *pheochromogenus*) seem less active. The test with Fehling's solution shows that only two groups, *Act. bobili* and *diastatochromogenus*, produce appreciable amounts of reducing sugars in the medium; the first species was also found by Waksman (11, 12) to produce invertase.

As a test for *proteolytic activities*, organisms were grown on the following solution: gelatin, 20 gm.; K_2HPO_4, 2 gm.; $MgSO_4$, 0.5 gm.; NaCl, 0.5 gm.; distilled water, 1,000 cc.; pH 7. The solution was used in portions of 25 cc. in 50-cc. Erlenmeyer flasks and incubated at 25°C. Four parallel flasks were used, of which two were analyzed after 10 days and two after 30 days. Ammonia-N was determined by distillation with MgO, formol-titrating N by the method of Sørensen.

Table 6 gives the results, together with records of the liquefaction of gelatin

TABLE 6

Proteolytic activity of actinomycetes in 2 per cent gelatin solution

SPECIES-GROUP	STRAIN	10 DAYS PER 25 CC. SOLUTION		30 DAYS PER 25 CC. SOLUTION		LIQUEFACTION OF	
		NH_4-N	Formol-titr. N	NH_4-N	Formol-titr. N	Gelatin	Blood serum
		mgm.	mgm.	mgm.	mgm.		
Actinomyces griseus	A. L. VIII.	3.7	18.8	14.7	34.4	Very rapid	+
	H. VI.	6.8	14.2	17.5	32.2		
	M. III.	4.0	15.3	17.7	31.9		
	V. III.	9.0	17.7	16.1	27.0		
Actinomyces griseoflatus	Hed. IV.	6.8	17.9	22.8	38.5	Very rapid	+
	A. XVIII.	7.4	16.2	21.4	34.7		
Actinomyces cellulosae	Sp. I.	3.7	16.1	14.7	27.3	Rapid	+
	A. V.	6.6	15.5	14.6	24.9		
	A. X.	3.9	16.8	13.7	27.0		
	A. L. III.	3.4	15.1	15.3	29.8		
Actinomyces olivaceus	B. XV.	1.6	11.3	6.0	28.4	Rapid	+
	L. II.	3.0	12.1	12.3	19.1		
	L. M. II.	2.5	15.1	5.6	28.9		
	W. aut.	2.7	14.2	5.8	25.3		
Actinomyces fulvissimus	H. I.	4.1	15.8	9.7	34.8	Fairly rapid	÷
	T. XIV.	0.9	7.8	4.2	24.2		
	H. X.	2.3	13.5	7.7	33.3		
Actinomyces violaceus-ruber	H. L. II	2.6	11.8	10.0	31.4	Rapid	÷
	H. L. IV.	2.8	17.2	9.0	46.9		
	V. G. III.	2.3	14.7	8.4	46.2		
Actinomyces roseus	B. V.	2.3	11.4	11.6	21.4	Slow	−
	R. IV.	2.0	11.4	8.8	18.9		
Actinomyces bobili	V. G.	1.4	9.0	3.3	19.1	Slow	÷
	W. aut.	3.2	13.3	9.0	34.7		
Actinomyces viridochromogenus	S. VI (?).	2.3	9.7	6.7	15.8	Slow	÷
	W. aut.	2.8	12.5	14.4	23.8		
Actinomyces erythrochromogenus	T. IV.	3.5	13.0	7.7	20.5	Slow	
Actinomyces pheochromogenus	H. b.	2.0	9.3	4.4	22.3	Slow	÷
Actinomyces diastatochromogenus	B. IV.	3.1	11.9	11.2	23.5	Slow	÷
	A. XVI.	3.0	10.4	10.9	19.4		
Actinomyces aureus (?)	L. S.	2.1	8.8	5.8	22.6	Slow	÷
Sterile solution	−	−	−	0.0	6.8	−	−

and blood serum. Although there is considerable variation among the strains within each single group, *Act. griseus*, *griseoflavus*, and *cellulosae* are seen to form a separate group of strains which split large amounts of protein, and a remarkably large proportion of this is present as ammonia-N; especially is this true of *Act. griseus*, which Waksman (11, 12) also found to be a very strongly proteolytic organism, and the closely related *Act. griseoflavus*. The next three groups of non-chromogenus actinomycetes, viz., *Act. olivaceus*, *violaceus-ruber*, and *fulvissimus* split about as much protein—two strains of *violaceus-ruber* most of all—but a distinctly smaller part of this is present as ammonia. Finally, the whole group of chromogenus actinomycetes (with one exception, the authentic strain of *Act. bobili*) shows a weaker proteolytic power and in most cases produces only small amounts of ammonia. The table further shows a fair parallelism between proteolytic power and rapidity of gelatin liquefaction, and, in agreement with the observations of Waksman (12), it shows that only the highly proteolytic strains are capable of liquefying blood serum.

RESISTANCE OF ACTINOMYCETES TO ACIDITY

All authors seem to agree that the actinomycetes as a whole are very sensitive toward acid reaction. Gillespie (5) showed that the hydrogen-ion concentration which checks the growth of *Act. scabies* in nutrient solution, is pH 4.8 to 5.2, and in accordance herewith Waksman and Joffe (17) found that *Act. violaceus ruber*, *scabies*, *griseus*, and *reticulus ruber* would produce a scant growth in solutions of pH 5, but not at pH 4.8; they further observed that when grown in a nutrient solution with dextrose as a source of carbon and ammonium sulfate as a source of nitrogen, nine species of actinomycetes would change the reaction from pH 5.8 to pH 4.4 to 4.6, one species (*Act. viridochromogenus*) even to pH 4.2. Waksman (14) further found that the limiting reaction of pH 4.8 to 5 also holds for *Act. scabies* when grown in sterile soil; some non-parasitic forms (*griseus*, *violaceus ruber*) seemed able to resist a slightly higher acidity.

An attempt was made here to differentiate between the groups of actinomycetes by determining the maximum hydrogen-ion concentration which they would tolerate, expressed as the degree of acidity arising in a physiologically acid medium. The following solution was used: dextrose, 20 gm.; NH_4Cl, 2 gm.; K_2HPO_4, 0.2 gm.; $MgSO_4$, 0.5 gm.; NaCl, 0.5 gm.; distilled water, 1,000 cc.; pH 6.5. Ten cubic centimeter portions in test tubes were used, and duplicate cultures were incubated for 15 days at 25°C., after which time the reaction was measured by means of the quinhydrone electrode.

The results, given in table 7, show some interesting features. The strains within each group seem to show far less variation in this than in any other respect here studied (the only serious discrepancy is shown by strain LII of the *olivaceus*-group), but the differences between the groups are far more considerable than those found by Waksman and Joffe (19). The groups of *Act. griseus*, *griseoflavus*, *cellulosae*, and *olivaceus* change the reaction only to

TABLE 7

Changes of reaction in cultures of actinomycetes on dextrose-NH_4Cl solution

Initial reaction pH 6.5

SPECIES-GROUP	STRAIN	FINAL pH	SPECIES-GROUP	STRAIN	FINAL pH
Actinomyces griseus	AL. VIII.	4.9–5.0	*Actinomyces violaceus-ruber*	HL. II.	4.6–4.6
	H. VI.	4.9–4.9		HL. III.	4.6–4.8
	V. III.	5.1–5.2		VB. III.	4.6–4.6
	W. aut.	4.9–4.9		W. aut.	4.4–4.5
Actinomyces griseoflavus	Hed. IV.	4.9–4.9	*Actinomyces roseus*	B. V.	3.7–3.8
	A. XVIII.	4.9–4.9		V. II.	3.6–3.7
Actinomyces cellulosae	Sp. I.	4.8–4.9	*Actinomyces bobili*	V. XII.	3.8–3.9
	A. V.	4.9–4.9		V. XIII.	3.7–3.8
	AL. III.	4.8–4.9		W. aut.	3.9–3.9
Actinomyces olivaceus	Sp. III.	5.0–5.1	*Actionomyces Halstedii* (?)	B. IX.	3.0–3.1
	L. II.	5.4–5.5		B. XIX.	3.4–3.5
	SD.	4.8–4.8			
	W. aut.	4.8–5.0	*Actinomyces viridochromogenus*	SVI (?)	4.1–4.2
				W. aut.	4.2–4.2
Actinomyces fulvissimus	H. I.	4.2–4.2			
	T. XIV.	4.2–4.2	*Actinomyces aureus* (?)	LS.	3.9–4.1
	R. V.	4.2–4.3			
	V. XXII	4.3–4.3	*Actinomyces pheochromogenus*	Hb.	4.6–4.7
Actinomyces erythrochromogenus	T. IV.	4.1–4.2			

TABLE 8

Growth of actinomycetes at pH 7.40 and 4.55

ORGANISM	GROWTH AT	
	pH 7.40	pH 4.55
Act. griseus, 3 strains	4–5	0
Act. griseoflavus, 2 strains	5	0
Act. cellulosae, 3 strains	4–5	0
Act. olivaceus, 3 strains	4–5	0
Act. fulvissimus, 3 strains	3–4	5
Act. violaceus-ruber, 3 strains	3–5	0
Act. roseus, 2 strains	2–3	3–4
Act. bobili, 2 strains	3–4	2–4
Act. Halstedii (?), 2 strains	4–5	4–5
Act. aureus (?)	4–5	4–5
Act. erythrochromogenus	3–3	3–4

pH 4.8 to 5.1—just the value which was found critical for the actinomycetes studied by Waksman and Joffe, and by Gillespie. *Act. violaceus-ruber* goes a little further, to pH 4.4 to 4.6, and the rest of the strains are able to resist still higher concentrations of hydrogen ions, one strain of *Act. Halstedii* (even acidifying the medium to pH 3. As a control on these figures a small experiment was run on the ability of the actinomycetes to induce growth at acid reaction. The following solution was used: starch, 10 gm.; asparagin, 2 gm.; K_2HPO_4, 2 gm.; $MgSO_4$, 0.5 gm.; NaCl, 0.5 gm.; distilled water, 1,000 cc. Since lack of time prevented the carrying out of an experiment over a wide range of pH values, only the original medium of pH 7.4 and a medium with reaction adjusted to pH 4.55 by means of hydrochloric acid were used. The arrangement of the experiment was as before. After 10 days' growth at 25°C. the results found in table 8 were noted.

They agree fully with the previous experiment: the groups which acidify the medium only to pH 4.8 to 5.0 all refuse to grow at pH 4.55, as does *Act. violaceus-ruber*, for which the pH region of 4.4 to 4.6 is just the critical value; but all the more resistant strains produce a more or less vigorous growth at this reaction. The limiting pH value thus seems to be of considerable value for characterizing the various species-groups.

CONCLUSIONS

As was mentioned in the preface, all investigators agree about the great variability of the actinomycetes, which may vary in their appearance, not only on different media at the same time, but also on the same medium at different periods of time, so much that it is hardly ever possible to obtain two strains that show entire agreement. However, as Waksman (11) points out, closer study will often show these variations to be quantitative rather than qualitative, so that it is possible to classify them in groups, the limits of which must still be rather vague, since they must generally be left to the judgment of each individual investigator. In the present work this great variability was fully recognized, but in spite of this, certain outstanding features remain, which may serve for characterization of the various species-groups. For instance, all the strains of the *griseus* and *griseoflavus* groups have their strong proteolytic power, especially characterized by an abundant ammonia formation; this was found true of freshly isolated strains (H. IV, V. III) as well as of strains which had been kept in culture for more than two years (A. L. VIII, A. XVIII, Hed. IV); also the characteristic colors of the aerial mycelia remained constant. Further, the pigment production, which generally is subject to immense variation, may in several cases prove a qualitatively constant feature; for instance, none of the "chromogenus" species were ever seen to lose their property of forming brown pigments in protein media during the three years over which the work was extended, and the strains of the *fulvissimus* group retained their formation of the typical

golden pigment during the same period, although the intensity and brightness of the color varied considerably on the different media. Also such features as the litmus-like pigment and the strong nitrate reduction in the *violaceus-ruber* group and the peculiar appearance of the vegetative mycelium in the *bobili* group proved most characteristic. Simply composed, synthetic media are certainly the most suitable for bringing out the characteristic features in the vast majority of the cases [it is quite possible that Lieske's (9) conviction of the impossibility of classifying the actinomycetes is due to the fact that he did not employ any such media], but instances may also be found, where complex organic media will give a characteristic growth, such as the very striking appearance of the *bobili* group on nutrient agar with glycerin. Finally the resistance towards acidity promises to be of value for separating the groups from one another and should be tested on a much larger number of strains than has been possible here. Although the majority of the strains isolated during the course of these experiments could not be identified and many of them not classified in groups, the author feels that this failure is due more to a lack of a sufficient number of representatives for the various groups and to a lack of sufficiently thorough study of both morphological and physiological characters than to an inherent impossibility of classifying these organisms.

SUMMARY

Counts were made of actinomycetes in 56 Danish soils of different character. The numbers arranged from none or a few thousands to about 13 millions to a gram of soil. Nearly all strongly acid soils—of pH less than 5—had very small numbers of actinomycetes. The highest numbers seemed most frequent in the pH interval of 6.8 to 8. The percentage of actinomycetes in the total microflora on plates varied from 0 to 73 per cent; this figure was very low in strongly acid peat soils; otherwise it did not show any correlation with the soil reaction. Apart from the reaction there seemed to be no correspondence between the general soil types and the numbers of actinomycetes.

Several strains of actinomycetes isolated from these soils could be recognized as belonging to the following species-groups:

Common organisms: *Act. griseus, cellulosae, olivaceus, bobili, diastatochromogenus.* Fairly common to rare organisms: *Act. griseoflavus, violaceus-ruber, roseus, erythrochromogenus, pheochromogenus.* A new species-group, *Actinomyces fulvissimus* n. sp., is described.

The majority of the strains could not be identified as belonging to definite groups. The different strains within the same species-group often show great differences in their physiological characters, such as pigment production, ability to utilize various carbohydrates, acid formation, nitrate reduction, and proteolytic activity although some of these characters remained relatively constant and appeared to have diagnostic value. Organic acids were some-

times formed from carbohydrates; this was always connected with nitrite production. The "chromogenous" forms had, upon the whole, a lower proteolytic power than the non-chromogenous. The ability of actinomycetes to resist acid reaction varied widely; the final acidity produced in a physiologically acid nutrient solution for each species-group was subject to less variation than most other physiological characters and may be of value for the characterization and identification of the different species-groups.

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